

FORM PTO-1390
(REV. 12-29-99)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

66040-9651

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

09/530663

INTERNATIONAL APPLICATION NO.
PCT/US98/24138INTERNATIONAL FILING DATE
12 November 1998PRIORITY DATE CLAIMED
12 November 1997

TITLE OF INVENTION GENETIC ENGINEERING OF LIGNIN BIOSYNTHESIS IN PLANTS

APPLICANT(S) FOR DO/EO/US Vincent Lee C. Chiang, Chung-Jui Tsai, Wen-Jing Hu

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

Small Entity Declaration

"Express Mail" mailing label number EL417145331US

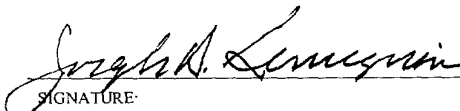
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date of my signature and is addressed to Box PCT, Assistant Commissioner for Patents, Washington, D.C. 20231.

Nancy Dragolovich

(Typed or printed name of person mailing paper or fee)

(Signature of person mailing paper or fee)

Date of Deposit 03 May 2000

U.S. APPLICATION NO. (if known, see 37 CFR 1.53) 09/530663		INTERNATIONAL APPLICATION NO. PCT/US98/24138		ATTORNEY'S DOCKET NUMBER 66040-9651	
17. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) : Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$970.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO. \$840.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$690.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$670.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$96.00 <div style="text-align: right;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div>				CALCULATIONS PTO USE ONLY	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 96.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	53 - 20 =	33	X \$18.00	\$ 594.00	
Independent claims	16 - 3 =	13	X \$78.00	\$ 1014.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$ 0	
TOTAL OF ABOVE CALCULATIONS =				\$ 1704.00	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				\$ 852.00	
SUBTOTAL =				\$ 852.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
TOTAL NATIONAL FEE =				\$ 852.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				\$	
TOTAL FEES ENCLOSED =				\$ 852.00	
				Amount to be refunded:	\$
				charged:	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>852.00</u> to cover the above fees is enclosed.					
b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.					
c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>133080</u> . A duplicate copy of this sheet is enclosed.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO Joseph A. Gemignani Michael Best & Friedrich LLP 100 East Wisconsin Avenue Milwaukee, WI 53202			 SIGNATURE: _____ Joseph A. Gemignani		
			NAME		
			19,482		
			REGISTRATION NUMBER		

09530563 071100

Under the Patent Act of 1952, no person is allowed to receive a collection of information unless it meets a need of the Government.

STATEMENT CLAIMING SMALL ENTITY STATUS (37 CFR 1.8(f) & 1.27(d)) - NONPROFIT ORGANIZATION		PTO/SB/11 (12-87) Docket Number (Optional) 66060-9651
Applicant, Patentee, or Identifier: <u>Chiang, et al.</u> Application or Patent No.: <u>PCT/US98/26138</u> Filed/Issued: <u>November 12, 1998</u> Title: <u>GENETIC ENGINEERING OF LIGNIN BIOSYNTHESIS IN PLANTS</u>		
I hereby state that I am an official empowered to act on behalf of the nonprofit organization identified below. NAME OF NONPROFIT ORGANIZATION <u>Board of Control of Michigan Technological University</u> ADDRESS OF NONPROFIT ORGANIZATION <u>1400 Townsend Drive, Houghton, MI 49931</u>		
TYPE OF NONPROFIT ORGANIZATION: <input checked="" type="checkbox"/> UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION <input type="checkbox"/> TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) and 501(c)(3)) <input type="checkbox"/> NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA (NAME OF STATE _____) (CITATION OF STATUTE _____) <input type="checkbox"/> WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA <input type="checkbox"/> WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA (NAME OF STATE _____) (CITATION OF STATUTE _____)		
I hereby state that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.8(e) for purposes of paying reduced fees to the United States Patent and Trademark Office regarding the invention described in: <input type="checkbox"/> the specification filed herewith with title as listed above. <input checked="" type="checkbox"/> the application identified above. <input type="checkbox"/> the patent identified above.		
I hereby state that rights under contract or law have been conveyed to and remain with the nonprofit organization regarding the above identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern, or organization having rights in the invention must file separate statements as to their status as small entities and that no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.8(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.8(d) or a nonprofit organization under 37 CFR 1.8(e). Each person, concern, or organization having any rights in the invention is listed below: <input checked="" type="checkbox"/> no such person, concern, or organization exists. <input type="checkbox"/> each such person, concern, or organization is listed below.		
I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))		
NAME OF PERSON SIGNING <u>William J. McGarry</u> TITLE IN ORGANIZATION OF PERSON SIGNING <u>Treasurer, Board of Control</u> ADDRESS OF PERSON SIGNING <u>1400 Townsend Drive, Houghton, Michigan 49931</u> SIGNATURE <u>William J. McGarry</u> DATE <u>3 May 2000</u>		

GENETIC ENGINEERING OF LIGNIN BIOSYNTHESIS IN PLANTS5 Cross-Reference to Related Applications

This is a continuation-in-part of U.S. application Serial No. 08/969,046, filed November 12, 1997, the disclosure of which is incorporated by reference herein.

Field of the Invention

10 The invention relates to genetically modifying plants, e.g., trees, through manipulation of the lignin biosynthesis pathway, and more particularly, to genetically modifying plants through the down regulation of 4-coumarate Co-enzyme A ligase (4CL) to achieve faster growth. Down regulation of 4CL may also achieve altered lignin content, and/or altered lignin structure, and/or altered
15 cellulose content, and/or altered disease resistance of the trees. Moreover, promoters of the 4CL genes are useful to drive gene expression specifically in xylem tissue or specifically in epidermal tissues.

Background of the Invention

Genetic engineering of plants to conform to desired traits has shifted the
20 emphasis in plant improvement away from the traditional breeding programs during the past decade. Although research on genetic engineering of plants has been vigorous, the progress has been slow.

The ability to make plants grow faster continues to be the top objective of many companies worldwide. The ability to genetically increase the optimal
25 growth of plants would be a commercially significant improvement. Faster growing plants could be used by all sectors of the agriculture and forest products industries worldwide.

Lignin, a complex phenolic polymer, is a major component in cell walls of secondary xylem. In general, lignin constitutes 25% of the dry weight of the
30 wood, making it the second most abundant organic compound on earth after cellulose. Although lignin plays an important role in plants, it usually represents an obstacle to utilizing biomass in several applications. For example, in wood

pulp production, lignin has to be removed through expensive and polluting processes in order to recover cellulose.

Thus, it is desirable to genetically engineer plants with reduced lignin content and/or altered lignin composition that can be utilized more efficiently.

- 5 Plants that could be genetically engineered with a reduced amount of lignin would be commercially valuable. These genetically engineered plants would be less expensive to pulp because, in essence, part of the pulping has already been performed due to the reduced amount of lignin. Further, plants with increased cellulose content would also be commercially valuable to the pulp and paper
10 industry.

Disease resistance in plants is also a desirable plant trait. The impact of disease resistance in plants on the economy of plant products industry worldwide is significant.

- Thus, what is needed is the identification and characterization of genes
15 useful to enhance plant growth, alter lignin content and/or structure in plants, alter cellulose content in plants, and/or provide or enhance disease resistance of plants.

Summary of the Invention

- The invention provides a method to genetically alter plants through the
20 down regulation (decrease) or inhibition of native (endogenous) 4-coumarate Co-enzyme A ligase (4CL) in that plant. Such down regulation of 4-coumarate Co-enzyme A ligase results in faster growth, and/or reduced lignin content, and/or altered lignin structure, and/or altered cellulose content, and/or altered disease resistance in the genetically altered plant. The invention also provides
25 for genetically engineered plants, e.g., transformed or transgenic plants, which have been altered to down regulate or inhibit native 4-coumarate Co-enzyme A ligase in the plant so as to achieve faster growth, and/or reduced lignin content, and/or altered lignin structure, and/or increased cellulose content, and/or increased disease resistance. Preferred genetically altered plants include trees,
30 e.g., angiosperms or gymnosperms, forage crops, and more preferably a forest tree, e.g., Populus. Preferred angiosperms include, but are not limited to, Populus, Acacia, Sweetgum, yellow poplar, maple and birch, including pure

lines and hybrids thereof. Preferred gymosperms include, but are not limited to, Pine, Spruce, Douglas-fir and hemlock.

The invention further provides a transgenic plant, the genome of which is augmented by a recombinant DNA molecule encoding 4-coumarate Co-enzyme
5 A ligase, or a recombinant DNA molecule comprising an antisense 4-coumarate Co-enzyme A ligase gene, or a fragment thereof. The recombinant DNA molecule is expressed so as to down regulate, decrease or inhibit lignin pathway 4-coumarate Co-enzyme A ligase.

The invention also provides an isolated and purified DNA molecule
10 comprising a DNA segment comprising a transcriptional regulatory control region of a 4-coumarate Co-enzyme A ligase gene. Preferably, the transcriptional regulatory region comprises a promoter. Tissue specific promoters of a 4-coumarate Co-enzyme A ligase gene can be used to manipulate gene expression in target tissue such as xylem and epidermal tissues, as
15 described hereinbelow. Preferably, the promoter is derived from aspen DNA. Therefore, the invention also provides an expression cassette comprising a transcriptional regulatory region of a 4-coumarate co-enzyme A ligase gene, a method of using the region to express a preselected DNA segment in a tissue-specific manner in plant cells, and a transgenic plant comprising the expression
20 cassette.

Also provided is a method to alter, e.g., enhance, plant growth. The method comprises introducing an expression cassette into cells of a plant, e.g., the cells of a tree, so as to yield genetically altered plant cells. The expression cassette comprises a recombinant DNA molecule, segment, or sequence,
25 comprising a 4-coumarate Co-enzyme A ligase gene, or a fragment thereof. Preferably, the 4-coumarate Co-enzyme A ligase gene, or fragment thereof, is in antisense orientation. The 4-coumarate Co-enzyme A ligase gene may be a homologous or heterologous 4-coumarate Co-enzyme A ligase gene. The transformed plant cells are regenerated to provide a genetically altered, e.g.,
30 transgenic, plant. The recombinant DNA is expressed in the cells of the regenerated, genetically altered plant in an amount that confers enhanced or accelerated growth to the regenerated, genetically altered plant relative to the

corresponding non-genetically altered plant. Preferably, the genetically altered plant is a tree. It is preferred that a genetically altered tree of the invention has an increase in height, leaf size, diameter and/or average internode length relative to the corresponding non-genetically altered tree.

- 5 Hence, the invention also provides for a genetically altered plant, the genome of which is augmented by a recombinant DNA molecule encoding 4-coumarate Co-enzyme A ligase, or a recombinant DNA molecule comprising an antisense 4-coumarate Co-enzyme A ligase gene, or fragment thereof, which plant has altered growth characteristics relative to the corresponding non-
- 10 genetically altered plant.

- Further provided is a method to genetically alter plants so as to change or alter their lignin structure. The method comprises introducing an expression cassette into cells of a plant, e.g., a tree, so as to yield genetically altered plant cells. The expression cassette preferably comprises an antisense recombinant
- 15 DNA molecule, segment or sequence comprising a 4-coumarate Co-enzyme A ligase gene, or a fragment thereof. The transformed plant cells are regenerated to provide a regenerated, genetically altered plant. The recombinant DNA is expressed in the cells of the regenerated, genetically altered plant in an amount that alters the lignin structure in the cells of the plant relative to the
- 20 corresponding non-genetically altered plant.

- Also provided is a method for altering the lignin content in a plant. The method comprises introducing an expression cassette comprising a recombinant DNA molecule comprising a 4-coumarate Co-enzyme A ligase gene operably linked to a promoter functional in a plant cell into the cells of a plant. The plant
- 25 cells are regenerated so as to yield a genetically altered plant. The recombinant DNA molecule is expressed in the cells of the regenerated plant in an amount effective to alter the lignin content in the plant cells. Preferably, the lignin content is reduced. Also preferably, the lignin content is reduced in a tissue-specific manner. In particular, a reduction in lignin content in forage crops is
- 30 useful as the digestability of these crops by ruminants is increased. Also preferably, the 4-coumarate Co-enzyme A ligase gene is in an antisense orientation relative to the promoter.

Further provided is a genetically altered, e.g., transgenic, plant having an altered lignin content in the plant cells. The plant comprises a recombinant DNA molecule comprising a nucleotide sequence encoding a plant 4-coumarate Co-enzyme A ligase operably linked to a promoter so that the recombinant DNA molecule is expressed in an amount effective to alter the lignin content of the plant.

Yet another embodiment of the invention is a method to alter, e.g., increase, the cellulose content in plants. The method comprises introducing an expression cassette into cells of a plant, e.g., a tree, so as to yield genetically altered plant cells. The expression cassette preferably comprises an antisense recombinant DNA molecule, segment or sequence comprising a 4-coumarate Co-enzyme A ligase gene, or a fragment thereof, operably linked to a promoter functional in a plant cell. The transformed plant cells are regenerated to provide a regenerated, genetically altered plant. The recombinant DNA is expressed in the cells of the regenerated, genetically altered plant in an amount that alters the cellulose content in plant. Thus, the invention further provides a genetically altered, e.g., transgenic, plant having an altered cellulose content.

The invention also provides a method to genetically alter plants to increase their disease resistance, e.g., to fungal pathogens. The method comprises introducing an expression cassette comprising a recombinant DNA molecule comprising a nucleotide sequence encoding a 4-coumarate Co-enzyme A ligase operably linked to a promoter functional in a plant cell into cells of a plant. The transformed plant cells are regenerated to provide a genetically altered plant. The recombinant DNA molecule is expressed in the cells of the regenerated, genetically altered plant in an amount effective to render the plant resistant to disease. Preferably, the recombinant DNA molecule is expressed in amount that decreases the amount of lignin in the plant and/or increases the amount of phenolic compounds which are toxic to fungal pathogens. Hence, the invention also provides a transgenic plant, which is substantially resistant to disease. The plant comprises a native 4-coumarate Co-enzyme A ligase gene, and a recombinant DNA molecule comprising a nucleotide sequence encoding 4-coumarate Co-enzyme A ligase operably linked to a promoter functional in a

plant wherein the recombinant DNA molecule is expressed in an amount effective to confer resistance to the transgenic plant.

Other features and advantages of the invention will become apparent to those of ordinary skill in the art upon review of the following drawings, detailed
5 description and claims.

Brief Description of the Drawings

Fig. 1 is a schematic of a phenylpropanoid pathway;

Fig. 2 is a diagram of Agrobacterium T-DNA gene construct pA4CL1;

Fig. 3 is a restriction map of genomic clone Pt4CL1g-4;

10 Fig. 4 is a restriction map of genomic clone Pt4CL2g-11;

Fig. 5 is a restriction map of subcloned pT4CL1 gene promoter p7Z-4XS;

Fig. 6 is a restriction map of subcloned pT4CL2 gene promoter pSK-
11HE;

Fig. 7 is an Agrobacterium T-DNA construct of Pt4CL1 promoter and
15 GUS fusion gene Pt4CL1p-GUS; and

Fig. 8 is an Agrobacterium T-DNA construct of Pt4CL2 promoter and
GUS fusion gene, Pt4CL2p-GUS.

Fig. 9 shows biosynthetic pathways to guaiacyl (coniferyl alcohol **9a**) and
syringyl (sinapyl alcohol **9b**) monolignols for the formation of guaiacyl-syringyl
20 lignin in wood angiosperms. Enzymes are indicated for each reaction step.
C4H, cinnamic acid 4-hydroxylase; C3H, 4-coumaric acid 3-hydroxylase;
COMT, caffeic acid *O*-methyltransferase; F5H, ferulic acid 5-hydroxylase; CCR,
cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase. Aspen 4CL
(Pt4CL1) converts 4-coumaric **2**, caffeic **3**, ferulic **4**, 5-hydroxyferulic **5**, and
25 sinapic **6** acids into their corresponding thioesters for the formation of feruloyl-
CoA **7a** and sinapoyl-CoA **7b**, leading to **9a** and **9b**, respectively.

Fig. 10. The effects of down-regulation of Pt4CL1 expression on Pt4CL1
activity and lignin accumulation in transgenic aspen. (A) Northern blot analysis
of Pt4CL1 transcript levels in control (lane C) and transgenic aspen (3, 4, 5, 6, 8,
30 and 9). Each lane contained 20 µg of total RNA extracted from developing
xylem and the blot was hybridized (Sambrook et al., Molecular Cloning: A
Laboratory Manual, Cold Spring Harbor Laboratory Press, NY (1989)) with

Pt4CL1 cDNA. (B) Pt4CL1 enzyme activities in developing xylem tissues.

Crude protein (40 µg) extracted from xylem tissue was assayed spectrophotometrically for Pt4CL1 activities with various hydroxylated cinnamic acids (Ranjeva et al., 1976). Error bars represent SD values of three replicates.

- 5 (C) Levels of lignin reduction in woody stem of transgenic lines as compared to the control, based on the lignin contents presented in Table 7. (D and E) Fluorescence microscopy showing transverse sections of the 20th internode from control (D) and transgenic line 6 (E). Lignin autofluorescence was visualized following UV-excitation at 365 nm.

- 10 Fig. 11 depicts regions of the HSQC spectra (NMR experiments were performed at 360 MHz on a Bruker DRX-360 using a narrow bore probe with inverse coil geometry (proton coils closest to the sample) and with gradients. Experiments used were standard Bruker implementations of gradient-selected inverse (¹H-detected) HSQC (Palmer et al., J. Magn. Reson. Ser. A, 111, 70
15 (1991)), HSQC-TOCSY (Braunschweiler et al., J. Magn. Reson., 53, 521 (1983)), and HMQC (Ruiz-Cabello et al., J. Magn. Reson., 100, 282 (1992)) along with the standard 1D ¹³C (proton-decoupled) and ¹H NMR experiments. TOCSY experiments used a 100 ms spin lock period; HMBC used either an 80 or a 100 ms long-range coupling delay.) of isolated milled wood lignins from (A)
20 control and (B) transgenic line 6. Structure assignments (Ralph et al., 1997) reveal the existence of some major structural units in both samples that are common to angiosperm lignin. The *erythro*-(δ_{Ca}/δ_{Ha} :75.4/6.05) and *threo*-(δ_{Ca}/δ_{Ha} :76.6/6.08) isomers of β -aryl ethers 10 are indicated. 5-5-Homo-coupling of coniferyl alcohol 9a involved in dibenzodioxocins 13 (δ_{Ca}/δ_{Ha} :85.3/4.94)
25 (Ralph et al., 1997) was not detected in either sample. Yellow contours are from intense methoxyl signals and light green contours form xylan residues. Other components (gray contours) in both lignin samples, not relevant or not identified, are commonly seen in many other angiosperm lignin preparations.

- Fig. 12 shows enhanced growth in transgenic aspen. (A) 10-Week-old
30 plants of control and four transgenic aspen grown in a greenhouse (ruler = 25 cm). (B) Control and transgenic leaves from the 10th internodes. (C to F) SE images of stem transverse sections of control [C (bar = 50 µm) and E (bar = 10

μm)] and transgenic line 6 [D (bar = 50 μm) and F (bar = 10 μm)]. (G) 2-week-old *ex vitro* rooted stem cuttings from control and transgenic aspen lines 5 and 6. Two cuttings from each line are shown. (H) Leaf upper epidermal cell area. Values represent the mean of at least 100 determinations per leaf. Sample SD was 15 to 20% of the mean for all determinations.

Before one embodiment of the invention is explained in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description of the preferred embodiment. The invention is capable of other embodiments and of being practiced or being carried out in various ways. Also, it is to be understood that the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting.

Detailed Description of the Invention

The invention pertains to genetically down regulating a lignin pathway 4-coumarate Co-enzyme A ligase (4CL) in a plant. Plants which have been genetically transformed to down regulate 4CL will hereafter be called transgenic plants. Such down regulation can result in faster growing plants. Such down regulation can also result in a reduction in the lignin content of the plants and/or altered lignin structure. Such down regulation can further result in increased cellulose content. Such down regulation may also result in increased disease resistance. Further, by using a specific 4CL promoter, targeted tissue-specific gene expression can be achieved in either the xylem or the epidermal tissues of the plant.

A. 4CL

Lignin is synthesized by the oxidative coupling of three monolignols (coumaryl, coniferyl and sinapyl alcohols) formed via the phenylpropanoid pathway as shown in Fig. 1. Reactions in the phenylpropanoid pathway include the deamination of phenylalanine to cinnamic acid followed by hydroxylations, methylations and activation of substituted cinnamic acids to coenzyme A (CoA) esters. The CoA esters are then reduced to form monolignols which are secreted from cells to form lignin.

The products of the phenylpropanoid pathway are not only required for the synthesis of lignin but also required for the synthesis of a wide range of aromatic compounds including flavonoids, phytoalexins, stilbenes and suberin.

- In the phenylpropanoid pathway, 4CL activates a number of cinnamic acid derivatives, including 4-coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid and sinapic acid. The resulting products, CoA esters, serve as substrates for entry into various branch pathways, such as lignin, flavonoids, phytoalexins, stilbenes and suberin. The esterification reactions catalyzed by 4CL require high energy and the reactions are not likely to occur without 4CL.
- 4CL is important in making a continuous flow of the lignin biosynthesis pathway. 4CL is also important because it is located at the branching points of the phenylpropanoid metabolism. 4CL is suggested to play a pivotal role in regulating carbon flow into specific branch pathways of the phenylpropanoid metabolism in response to stages of development and environmental stress.
- The basic properties of 4CL are quite uniform. 4CL depends on ATP as a cosubstrate and requires Mg^{2+} as a cofactor. The optimal pH for 4CL ranges from pH 7.0 to 8.5 and the molecular weight of 4CL isoforms from various plant species ranges from 40 kD to 75 kD. Most 4CLs have high affinity for substituted cinnamic acids. 4CL has the highest activity with 4-coumaric acid.
- 4CL cDNA sequences have been reported for parsley, potato, soybean, rice, loblolly pine, *Arabidopsis*, *Lithospermum*, *Vanilla* and tobacco. 4CL genes have been isolated and sequenced for parsley, rice, potato and loblolly pine. The analysis of 4CL cDNAs and genes indicates that 4CL is encoded by multiple/divergent genes in rice, soybean, and *Lithospermum*, very similar genes in parsley, potato, tobacco, and loblolly pine, and a single gene in *Arabidopsis*.
- Two similar 4CL cDNAs in parsley, potato and tobacco have been shown to be expressed at similar level in response to environmental stress and during different developmental stages. Two distinct 4CL cDNAs in soybean and *Lithospermum* have shown different expression levels when pathogens or chemicals were applied to the cell cultures. It appears that the expression of the 4CL genes is developmentally regulated and inducible by many environmental

stresses at the transcription level. 4CL promoters have been isolated and sequenced for parsley, rice and potato.

Alignment of deduced amino acid sequences of cloned plant 4CL sequences reveals two highly conserved regions. The first conserved region
5 (LPYSSGTTGLPK; SEQ ID NO:7), proposed to designate a putative AMP-binding region, consists of a serine/threonine/glycine-rich domain followed by a proline/lysine glycine triplet. The second conserved region (GEICIRG; SEQ ID NO:8) contains one common Cys residue. The amino acid sequences of 4CL from plants contain a total of five conserved Cys residues.

10 The description of the invention hereafter refers to the tree species aspen, and in particular quaking aspen (*Populus tremuloides* Michx), when necessary for the sake of example. However, it should be noted that the invention is not limited to genetic transformation of trees such as aspen. The method of the present invention is capable of being practiced for other plant species, including
15 for example, other angiosperm, and other gymnosperm forest plants species, legumes, grasses, other forage crops and the like.

Preferably, the 4CL down regulation is accomplished through transformation with a homologous 4CL sequence in an antisense orientation. However, it should be noted that a heterologous antisense 4CL sequence could
20 be utilized and incorporated into a plant species to down regulate 4CL if the heterologous 4CL gene sequence has a high nucleotide sequence homology or identity of at least about 70%, more preferably at least about 80%, and more preferably at least about 90%, to the endogenous (native) 4CL gene sequence of that plant species, e.g., a tree species.

25 In addition, plants transformed with a sense 4CL sequence may also cause a sequence homology-based cosuppression of the expression of the transgene and endogenous 4CL gene, thereby achieving down regulation of 4CL in these plants.

B. Isolation of 4CL cDNAs

30 The present invention utilizes a homologous 4CL sequence to genetically alter plants. The example described below utilizes a cDNA clone of the quaking aspen 4CL gene to genetically alter quaking aspen.

Two 4CL cDNAs, Pt4CL1 and Pt4CL2, have been isolated from quaking aspen. Pt4CL1 cDNA is lignin pathway-specific and is different from Pt4CL2 cDNA, which is involved in flavonoid synthesis. It should be noted that the methods described below are set forth as an example and should not be considered limiting. The sequences of these 4CL cDNA clones are available from Genbank, Accession Nos. AF041049 and AF041050.

Pt4CL1 and Pt4CL2 genomic clones including their 5' -end regulatory promoter sequences were also isolated. The promoter of Pt4CL 1 (Pt4CL1p) directs xylem tissue-specific gene expression in a plant, whereas the promoter of Pt4CL2 (Pt4CL2p) drives the expression of genes specifically in epidermal tissues of stem and leaf of a plant. These tissue specific promoters will be discussed in more length below.

Young leaves and shoot tips are collected from greenhouse-grown quaking aspen (*Populus tremuloides* Michx). Differentiating xylem is collected from three to four year old quaking aspen. The bark is peeled from the tree exposing the developing secondary xylem on the woody stem. Developing secondary xylem is scraped from the stem and bark with a razor blade and immediately frozen in liquid nitrogen until use.

Total RNA is isolated from the young leaves, shoot tips, and xylem following the method of Bugos et al., Biotechniques 19(5):734-737 (1995). Poly(A)⁺ RNA is purified from total RNA using Poly(A)⁺ mRNA Isolation Kit from Tel-test B, Inc. A unidirectional Lambda gt22 expression cDNA library was constructed from the xylem mRNA using Superscript λ System from Life Technologies, Inc. and Gigapack Packaging Extracts from Stratagene. The Pt4CL1 cDNA was obtained by screening the cDNA library with a ³²P-labeled parsley 4CL cDNA probe. The parsley 4CL cDNA (pc4CL2) has Genbank Accession No. X13325.

The Pt4CL2 cDNA was obtained by RT-PCR. The reverse transcription of total RNA isolated from shoot tips was carried out using the Superscript II reverse transcriptase from Life Technologies. Two sense primers (R1S, 5'-TTGGATCCGGIACIACIGGIYTICCIAARGG-3'; SEQ ID NO:9 and H1S, 5'-TTGGATCCGTIGCICARCARGTIGAYGG-3'; SEQ ID NO:10) were designed

around the first consensus AMP-binding region of 4CL that was previously discussed. One antisense primer (R2A, 5'-ATGTCGACCICKDATRCADATYTCICC-3'; SEQ ID NO:11) was designed based on the sequence of the putative catalytic motif GEICIRG (SEQ ID NO:12).

- 5 One fifth of the reverse transcription reaction (4 μ l) is used as the template in a 50 μ l PCR reaction containing 1X reaction buffer, 200 μ M each deoxyribonucleotide triphosphate, 2 μ M each R1S and oligo-dT (20 mer) primers, and 2.5 units of *Taq* DNA polymerase. The PCR reaction mixture was denatured at 94°C for 5 minutes followed by 30 cycles of 94°C/45 seconds, 10 50°C/1 minute, 72°C/90 seconds and is ended with a 5 minute extension at 72°C. 2 μ l of the PCR amplification products were used for a second run PCR re-amplification using primers H1S and R2A. A 0.6 kb PCR fragment was cloned using the TA Cloning Kit from Invitrogen and used as a probe to screen an aspen genomic library to obtain the Pt4CL2 genomic clone. Two primers 15 (2A, 5'-TCTGTCTAGATGATGTCGTGGCCACGG-3'; SEQ ID NO:13 and 2B, 5'-TTAGATCTCTAGGACATGGTGGTGGC-3'; SEQ ID NO:14) were designed based on the genomic sequence of Pt4CL2 around the deduced transcription start site and the stop codon. These primers were used to clone Pt4CL2 cDNA by RT-PCR, as described above using total RNA isolated from 20 shoot tips.

The DNA sequences of Pt4CL1 and Pt4CL2 cDNA were determined using Δ *Taq* Cycle Sequencing kit from Amersham.

- The Pt4CL1 cDNA has an open reading frame of 1605 bp which encodes a polypeptide of 535 amino acid residues with a predicted molecular weight of 25 58.498 kd and pI of 5.9. The nucleotide sequence of the aspen 4CL cDNA clone Pt4CL1 is set forth as SEQ ID NO:1. The deduced amino acid sequence for the aspen 4CL1 protein is set forth as SEQ ID NO:2.

- The Pt4CL2 cDNA has an open reading frame of 1710 bp which encodes a polypeptide of 570 amino acid residues with a predicted molecular weight of 30 61.8 kd and pI of 5.1. The nucleotide sequence of the aspen 4CL cDNA clone Pt4CL2 is set forth as SEQ ID NO:3. The deduced amino acid sequence for the aspen 4CL2 protein is set forth as SEQ ID NO:4.

The aspen Pt4CL1 cDNA shares a 55-69% identity at the nucleotide level and 57-76% identity at the amino acid level with previously reported 4CL cDNAs and genes, whereas the Pt4CL2 cDNA shares a 60-71% identity at the nucleotide level and 58-73% at the amino acid level with other 4CL cDNAs and
5 genes as set forth in the following table.

Table 1: Comparison of Pt4CL1 and Pt4CL2 nucleotide and predicted amino acid sequences to each other and other full length 4CL sequences.

Gene	Comparison with Pt4CL1 (%)			Comparison with Pt4CL2 (%)		
	DNA Identity	Protein Identity	Protein Similarity	DNA Identity	Protein Identity	Protein Similarity
Pt4CL1				61.3	63.4	72.7
Pt4CL2	61.3	63.4	72.7			
Le4CL1	64.5	70.7	78.1	61.8	64.6	73.4
Le4CL2	60.1	57.3	67.7	71.1	73	77.5
Nt4CL1	66	74.8	83.1	61.5	65.3	74.4
Nt4CL2	64.1	75	82.9	62.1	66.8	76
Os4CL1	59.2*	59.8	70.2	59.6*	57.7	69.5
Os4CL2	54.9	57.7	67.3	63.9	66.5	73.8
Pc4CL1	65.1	71.2	79.6	62	64.3	73.5
Pc4CL2	65	71.4	79.6	62.9	64.5	73.5
Ptd4CL1	66.6	73.7	82.2	64.5	66.6	75.8
Ptd4CL2	67	74.2	81	63.4	64.7	73.3
At4CL	63.7	69.9	78.7	62.4	61.1	70.2
Lp4CL	60.1	64	73.9	62.3	67.9	77.8
St4CL1	69.1*	74	81.4	62.2*	65.3	74.5
Vp4CL	65.2	75.5	81.6	61.5	66.5	74.1

Pt4CL1 and Pt4CL2: aspen 4CL

Le4CL1 and Le4CL2: lithospermum erythrorhizon 4CL

Nt4CL1 and Nt4CL2: tobacco 4CL

Os4CL1 and Os4CL2: rice 4CL (*DNA sequence compared to Os4CL1 coding region only)

Pc4CL1 and Pc4CL2: Parsley 4CL

Ptd4CL1 and Ptd4CL2: hybrid poplar 4CL

At4CL: Arabidopsis 4CL

Lp4CL: lobolly pine 4CL

St4CL: potato 4CL (*DNA sequence compared to coding region only)

Vp4CL: vanilla 4CL

In a study to characterize lignification in aspen stems, it was observed that the lignin composition in the top four internodes (referred to as top internodes hereafter) was different from that in the fifth and subsequent internodes, suggesting the involvement of developmentally regulated differential expression of lignin pathway genes during the transition from primary to secondary growth in aspen stem. To investigate whether this transition regulates differential expression of 4CL gene members, 4CL genes were cloned from top and lower (6th-10th) internodes and secondary-developing xylem tissue of aspen stems. Nucleotide sequence analysis revealed that clones derived from lower internodes were identical to Pt4CL1, whereas clones isolated from top internodes could be divided into two groups (T1 and T2). Clones in Group T1 were found identical to Pt4CL1. Clones in group T2 shared 60-75% sequence homology with other plant 4CL genes but were distinct from Pt4CL1 cDNA and designated as Pt4CL2-600. These results together with Northern hybridization analysis suggested that Pt4CL2-600 represents a fragment of another aspen 4CL gene expressed in top internodes.

The results of sequence analysis, phylogenetic tree and genomic Southern blot analysis indicate that Pt4CL1 and Pt4CL2 cDNAs encode two distinct 4CLs that belong to two divergent gene families in aspen. The deduced amino acid sequence for the Pt4CL2 protein contains a longer N-terminal sequence than the Pt4CL1 protein but shows profound similarity in the central and C-terminal portions of protein to the Pt4CL1 protein.

Pt4CL1 and Pt4CL2 cDNAs display distinct tissue-specific expression patterns. The Pt4CL1 sequence is expressed highly in the secondary developing xylem and in the 6th to 10th internodes whereas the Pt4CL2 sequence is expressed in the shoot tip and leaves. These tissue-specific expression patterns were further investigated by fusing promoters of Pt4CL1 and Pt4CL2 genes to a GUS reporter gene. The tissue specific promoters for Pt4CL1 and Pt4CL2 are discussed in more length below.

The substrate specificity of Pt4CL1 and Pt4CL2 is also different from each other as determined using recombinant proteins produced in *E. coli*.

Pt4CL1 utilized 4-coumaric acid, caffeic acid, ferulic acid and 5-hydroxyferulic acid as substrates. Pt4CL2 showed activity for 4-coumaric acid, caffeic acid and ferulic acid but not to 5-hydroxyferulic acid.

Specifically, Pt4CL1 and Pt4CL2 were used to construct expression
5 vectors for *E. coli* expression. The substrate specificity of Pt4CL1 and Pt4CL2 was tested using fusion proteins produced in *E. coli*. Two plasmids, pQE/4CL1 and pQE/4CL2, were constructed in which the coding regions of Pt4CL1 and Pt4CL2, respectively, were fused to N-terminal His tags in expression plasmids pQE-31 and pQE-32 (QIAGEN, Chatsworth, CA). The recombinant proteins of
10 Pt4CL1 and Pt4CL2 produced by *E. coli* were approximately 60 kD and 63 kD, respectively.

The two recombinant proteins were tested for their activity in utilizing cinnamic acid derivatives. Pt4CL1 recombinant protein showed 100, 51, 72, 19 and 0% relative activity to 4-coumaric acid, caffeic acid, ferulic acid, 5-
15 hydroxyferulic acid and sinapic acid, respectively. Pt4CL2 recombinant protein exhibited 100, 31, 26, 0 and 0% relative activity to 4-coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid and sinapic acid, respectively. Neither recombinant protein showed detectable activity to sinapic acid.

The results of the tissue-specific expression pattern and substrate
20 specificity suggests that in addition to the general function of 4CL, Pt4CL1 apparently is more related to lignin synthesis in the xylem tissue and Pt4CL2 apparently is more likely involved in flavonoid synthesis and UV protection.

It should be noted that the isolation and characterization of the Pt4CL1 and Pt4CL2 cDNA clones is described in Kawaoka et al., Proceedings of the 6th
25 International Conference on Biotechnology in the Pulp and Paper Industry, Vienna, Austria (1995); and in Hu, Wen-Jing, Isolation and Characterization of 4-coumarate: Coenzyme A Ligase cDNAs and Genes from Quaking Aspen (*Populus tremuloides* Michx), Ph.D. Dissertation, Michigan Technological University, Houghton, Michigan (1997); and Tsai et al., Plant Physiol., 117, 101
30 (1998).

C. Transformation and Regeneration

Several methods for gene transformation of plant species with the 4CL sequence are available such as the use of *Agrobacterium*, electroporation, particle bombardment with a gene gun or microinjection.

5 Preferably, a 4CL cDNA clone is positioned in a binary expression vector in an antisense orientation under the control of double cauliflower mosaic virus 35S promoter. The vector is then preferably mobilized into a strain of *Agrobacterium* species such as *tumefaciens* strain C58/pMP90 and is used as the DNA delivery system due to its efficiency and low cost.

10 For example, with reference to Fig. 2, the binary expression pA4CL1 used for plant transformations is shown. Specifically, the Pt4CL1 cDNA is inserted in an antisense orientation into *Pac* I and *Bam*H I sites between the double CaMV 35S/AMV RNA4 and the 3' terminator sequence of the nopaline synthase gene in a binary cloning vector pA4CL1 (Fig. 2). The binary vector
15 containing hygromycin phosphotransferase (*HPT*) gene is modified from pBin 19. The gene construct pA4CL1 is available from Michigan Technological University, Institute of Wood Research, Houghton, Michigan.

The binary vector construct is mobilized into *Agrobacterium tumefaciens* using the freeze-thaw method of Holsters et al., Mol. Gen. Genet. 163: 181-187
20 (1978). For the freeze-thaw method, 1.5 ml of overnight cultures *Agrobacterium tumefaciens* strain C58/pMP90 is pelleted at 5000 x g for 3 minutes at 4°C and suspended in 1 ml of ice cold 20 mM CaCl₂. To the suspension is added 10 µl binary vector DNA (from an alkaline lysis miniprep) and mixed by pipetting. The microcentrifuge tube is then frozen in liquid nitrogen for 5
25 minutes and thawed at 37°C for 5 minutes. After being cooled on ice, 1 ml of LB is added and the mixture is incubated at 28°C for 2 hours with gentle shaking. 200 µl of the cells is spread onto LB plates containing gentamycin and kanamycin and incubated at 28°C for 2 days. Colonies grown on the selection
30 plates are randomly picked or miniprep and restriction enzyme digestion analysis is used to verify the integration.

The resulting binary vector containing *Agrobacterium* strain is used to transform quaking aspen according to Tsai et al., Plant Cell Rep. 14: 94-97 as set forth below.

Explants of young leaves from cuttings of aspen are obtained by cutting
5 leaf disks of approximately 7 mm square from the young leaves along the midrib of the leaves. The explants are surface sterilized in 20% commercial bleach for 10 minutes followed by rinsing 3 times with sterile distilled, deionized water.

All of the culture media used includes the basal medium of woody plant medium (WPM) as described in Lloyd et al., Proc. Int. Plant Prop. Soc. 30: 421-
10 437 (1980) and supplemented with 2% sucrose. 650 mg/L calcium gluconate and 500 mg/L MES are added as pH buffers as described in Tsai et al., Plant Cell Reports, 1994. All culture media is adjusted to pH 5.5 prior to the addition of 0.75% Difco Bacto Agar and then autoclaved at 121 °C and 15 psi for 20 minutes. Filter sterilized antibiotics are added to all culture media after
15 autoclaving. All culture media are maintained at $23 \pm 1^\circ\text{C}$ in a growth chamber with 16 hour photoperiods ($160 \mu\text{E} \times \text{m}^{-2} \times \text{S}^{-1}$) except for callus induction (as will be described later) which is maintained in the dark.

The sterilized explants are then inoculated with the mobilized vector with an overnight-grown agrobacterial suspension containing 20 μM acetosyringone.
20 After cocultivation for 2 days, the explants are washed in 1 mg/ml claforan and ticarcillin for 2 hours with shaking to kill *Agrobacterium*. The explants are blotted dry with sterile Whatman No. 1 filter paper and transferred onto callus induction medium containing 50 mg/L kanamycin and 300 mg/L claforan to induce and select transformed callus. The callus induction medium is the basal
25 medium with the addition of 6-benzyladenine (BA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) at concentrations of 0.5 mg/L and 1 mg/L, respectively, to induce callus.

The kanamycin-resistant explants are then subcultured on fresh callus induction media every two weeks. Callus formation occurs after approximately
30 four weeks. Formed calli are separated from the explant and subcultured periodically for further proliferation.

When the callus clumps reach approximately 3 mm in diameter, the callus clumps are transferred to shoot regeneration medium. The shoot regeneration medium is the basal medium containing 50 mg/L kanamycin, 0.5 mg/L thidiazuron (TDZ) as a plant growth regulator and claforan at 300 mg/L to
 5 kill *Agrobacterium*. Shoots were regenerated about 4 weeks after callus is transferred to regeneration medium.

As soon as the shoots are regenerated, they are immediately transferred to hormone-free elongation medium containing 50 mg/L kanamycin and, whenever necessary, claforan (300 mg/L), to promote elongation. Green and healthy
 10 shoots elongated to 2-3 cm in length are excised and planted separately in a hormone-free rooting medium containing 50 mg/L kanamycin. The efficient uptake of kanamycin by shoots during their rooting stage provides the most effective selection for positive transformants. Transgenic plants are then transplanted into soil medium of vermiculite:peatmoss:perlite at 1:1:1 and grown
 15 in the greenhouse.

The above described transformation and regeneration protocol is readily adaptable to other plant species. Other published transformation and regeneration protocols for plant species include Danekar et al., Bio/Technology 5:587-590 (1987); McGranahan et al., Bio/Technology 6:800-804 (1988);
 20 McGranahan et al., Plant Cell Reports 8:512-616 (1990); Chen, Ph.D. Thesis, North Carolina State University, Raleigh, North Carolina (1991); Sullivan et al., Plant Cell Reports 12:303-306 (1993); Huang et al., In Vitro Cell Dev. Bio. 4:201-207 (1991); Wilde et al., Plant Physiol. 98:114-120 (1992); Minocha et al., 1986 Proc. TAPPI Research and Development Conference, TAPPI Press,
 25 Atlanta, pp. 89-91 (1986); Parsons et al., Bio/Technology 4:533-536 (1986); Fillatti et al., Mol. Gen. Genet 206:192-199 (1987); Pythoud et al., Bio/Technology 5:1323-1327 (1987); De Block, Plant Physiol. 93:1110-1116 (1990); Brasileiro et al., Plant Mol. Bio 17:441-452 (1991); Brasileiro et al., Transgenic Res. 1:133-141 (1992); Howe et al., Woody Plant Biotech., Plenum
 30 Press, New York, pp. 283-294 (1991); Klopfenstein et al., Can. J. For. Res. 21:1321-1328 (1991); Leple et al., Plant Cell Reports 11:137-141 (1992); and Nilsson et al., Transgenic Res. 1:209-220 (1992).

D. Phenotype Changes

The results of the transformation can be confirmed with conventional PCR and Southern analysis. Transferring 4CL cDNA in an antisense orientation down regulates 4CL in the plant. Expression of the 4CL has been found to be
5 blocked up to 96 percent of 4CL enzyme activity in some transgenic plants.

In the aspen example, after acclimation, the transgenic aspen displayed an unusual phenotype, including big curly leaves, thick stem diameter, longer internodes, more young leaves in the shoot tip and a red pigmentation in the petioles extending into midvein leaves. Red coloration of the developing
10 secondary xylem tissues is observed after peeling of the bark in the transgenic plants.

E. Accelerated Growth

Down regulation of 4CL altered growth of the transgenic plants. For example, transformation with an antisense 4CL sequence accelerated the growth
15 of the plant. Enhanced growth is markedly noticeable at all ages. In particular the transgenic trees showed enhanced growth in the form of thicker stems and enlarged leaves as compared to control plants. These characteristics are retained in the vegetative propagules of these transgenic trees. Table 2 sets forth exemplary data with respect to several lines of transgenic quaking aspen grown
20 in the greenhouse after eight months. Volume represents the overall quantitative growth of the plant.

Table 2: Growth Measurement for Control and Transgenic Plants

Plant #	Height (cm)	Diameter (cm)*	Volume (cm ³)*	Average Length of Internode (cm)
Control 1	247.7	1.08	75.6	2.6
Control 2	250.2	1.01	66.8	2.8
11-1	304.8	1.15	105.5	3.1
11-2	248.9	1.01	66.4	3.4
11-3	241.3	0.84	44.6	3.2
11-4	288.3	0.94	66.7	3.4
11-5	246.4	0.92	54.6	3.3
11-7	226.7	1.13	75.7	3.4
11-8	289.6	1.16	102.0	3.3
11-9	287.0	1.76	232.6	4.3
11-10	252.7	0.83	45.6	3.1
11-11	247.7	0.86	48.0	3.5
12-1	247.7	1.1	78.4	2.7
12-2	199.4	0.96	48.1	2.5
12-6	294.6	0.92	65.2	3.2
16-1	227.3	0.95	53.7	2.8
16-2	278.1	0.97	68.5	3.4
16-3	265.4	1.09	82.5	3.5
17-2	243.8	0.89	50.5	2.6

* at 10 cm above ground

The averages for height, diameter, volume and average length between internodes for the control plants are as follows:

Height (cm)	248.95
Diameter (cm)	1.045
Volume (cm ³)	71.2
Ave. Length of Internodes (cm)	2.7

With respect to height alone, for those transgenic plants (11-1, 11-4, 11-8, 11-9, 12-6, 16-2, 16-3) having a statistically larger height than the control plants, the average height was 286.83 cm as compared to the control plant average height of 248.95 cm.

With respect to diameter alone, for those transgenic plants (11-1, 11-7, 11-8, 11-9) having a statistically larger diameter than the control plants, the average diameter was 1.30 cm as compared to the control plant average diameter of 1.045 cm.

- 5 With respect to volume alone, for those transgenic plants (11-1, 11-8, 11-9, 12-1, 16-3) having a statistically larger volume than the control plants, the average volume was 120.2 cm³ as compared to the control plant average volume of 71.2 cm³.

- 10 With respect to average length of internodes alone, for those transgenic plants (11-1, 11-2, 11-3, 11-4, 11-5, 11-7, 11-8, 11-9, 11-10, 12-6, 16-2, 16-3) having a statistically larger average length of internodes than the control plants, the average length of internodes was 3.39 cm as compared to the control plant average length of internodes of 2.7₀ cm.

- 15 As demonstrated in Table 2, while there are variations in growth among the transgenic plants, the average length of the internodes for the transgenic plants is consistently and significantly higher than that of the control plants. Moreover, there is also faster root initiation, and alterations, e.g., an increase, in root fresh weight and length, i.e., enhanced root growth. Variations in the growth of the transgenic plants is normal and to be expected. Preferably, a
20 transgenic plant with a particular growth rate is selected and this plant is vegetatively propagated to produce an unlimited number of clones that all exhibit the identical growth rate.

F. Lignin

- 25 Down regulation of lignin pathway 4CL results in production of plants with reduced lignin content.

The following table shows the reduction of lignin content and 4CL enzyme activity in several transgenic aspen which were transformed with a homologous antisense 4CL sequence.

Table 3: Characterization of Transgenic Aspen Plants Harboring Antisense 4CL Sequence

Transgenic Plant #	Lignin Content % Based on Wood Weight	% Lignin Reduction	%4CL Enzyme Activity*	% 4CL Enzyme Activity Reduction
Control	21.4	0.0	868	0
11-1	20.5	4.2	1171	-25
11-2	19.2	10.3	515	45
11-3	20.9	2.3	922	6
11-4	19.7	7.9	1032	-19
11-5	19.7	7.9	691	20
11-7	19.9	7.0	578	38
11-8	20.2	5.6	694	20
11-9	20.4	4.7	806	14
11-10	19.4	9.3	455	51
11-11	20.4	4.7	726	22
12-1	12.8	40.2	49	95
12-2	12.6	41.1	62	93
12-3	11.9	44.4	61	94
12-6	19.8	7.5	786	16
16-1	12.8	40.2	35	96
16-2	20.6	3.7	780	17
16-3	21.0	1.9	795	15
17-1	20.5	4.2	855	9
17-2	21.4	0.0	925	1

*activity is expressed as pkat/(mg protein) using 4-coumaric acid as the substrate

Lignin content was determined according to Chiang and Funaoka (1990) *Holzforschung* 44:147-155. 4CL enzyme activity was determined according to Ranjeva et al. (1976), *Biochimie* 58:1255-1262.

The data in Table 3 demonstrates a correlation between down regulation of 4CL and reduction in lignin content. Transgenic plants with reduced lignin content have an altered phenotype in that the stem is more elastic to the touch or less curly.

It should also be noted that for those transgenic plants (12-1, 12-2, 12-3 and 16-1) with the approximately 40% reduction in lignin content and the corresponding approximately 95% reduction in 4CL enzyme levels, all of those transgenic plants had a consistent deep red coloration in the wood of the plant.

- 5 Accordingly, the deep red color can be used as an identifier of reduced lignin content.

Down regulation of lignin pathway 4CL can also result in production of plants with an altered lignin structure. Based upon thioacidolysis (Rolando et al. (1992) Thioacidolysis, Methods in Lignin Chemistry, Springer-Verlag, Berlin, 10 pp. 334-349) of plants 12-3 and 16-1, coniferyl alcohol and sinapyl alcohol lignin units are significantly reduced in these two plants as compared to the control tree, as shown in the following table.

Table 4: Altered Lignin Structure

15	Plant #	Coniferyl Alcohol Units *	Sinapyl Alcohol Units*
	Control	733	1700
	12-3	283	592
	16-1	247	445

*micro-mole/g of lignin

- 20 The alteration of the frequency of the structural units in lignin of these transgenic plants is evidence that the overall structure of lignin in these plants has been genetically altered.

G. Cellulose Content

- Down regulation of lignin pathway 4CL can result in increased cellulose 25 content of the transgenic plants. Analysis of control and transgenic aspen for carbohydrate content demonstrate a higher cellulose content in the transgenic plants than the control plants. Particularly, the transgenic plants that have over 40% lignin reduction have about 10-15% higher cellulose content than the control. Data is set forth in the following tables for trees that were transformed 30 with homologous 4CL in an antisense orientation:

Table 5: Analysis of Carbohydrate Components in Transgenic and Control Aspen

Plant #	Glucan	Arabinan	Galactan	Rhamnan	Xylan	Mannan
Control	44.23%	0.47%	0.79%	0.37%	17.19%	1.91%
11-2	49.05%	0.36%	1.05%	0.38%	15.34%	2.04%
11-9	45.95%	0.40%	0.80%	0.37%	17.12%	1.83%
11-10	47.49%	0.43%	0.99%	0.40%	16.24%	2.35%
12-3	50.83%	0.55%	1.24%	0.48%	17.25%	1.77%
16-1	48.14%	0.56%	1.07%	0.48%	19.14%	1.58%
16-2	46.55%	0.34%	0.82%	0.37%	16.75%	2.31%

Table 6: Comparison of Lignin and Cellulose (glucan) Contents in Transgenic and Control Aspen

Plant #	Lignin		Cellulose	
	Content % on Wood	% Reduction	Content % on Wood	% Increase
Control	21.4	0	44.23	0
11-2	19.2	10.3	49.05	10.9
11-9	20.4	4.7	45.95	3.9
11-10	19.4	9.3	47.49	7.4
12-3	11.9	44.5	50.83	14.9
16-1	12.8	40.2	48.14	8.8
16-2	20.6	3.7	46.55	5.2
11-6	18.6	13.1	45.98	3.8
12-1	12.5	40.2	48.35	9.3
12-2	12.6	41.1	49.74	12.5
12-5	14.4	32.7	45.58	3.1

The procedure for carbohydrate analysis utilized is as follows. About 100 mg of milled woody tissue powder with sizes that pass a 80-mesh screen was hydrolyzed with 1 mL of 72% (W/W) H₂SO₄ for 1 hr at 30°C. Samples were then diluted to 4% (W/W) H₂SO₄ with distilled water, fucose was added as

an internal standard, and a secondary hydrolysis was performed for 1 hr at 121 °C. After secondary hydrolysis, the sugar contents of the hydrolysates are determined by anion exchange high performance liquid chromatography using pulsed amperometric detection. Sugar contents are expressed as % of the weight of the woody tissue used. The above procedures are similar to those in a publication by Pettersen and Schwandt, J. Wood Chem & Technol. 11:495-501 (1991).

H. Increased Disease Resistance

Down regulation of lignin pathway 4CL can result in altered levels of phenylpropanoids or secondary metabolites that display antimicrobial activity. Thus, transgenic plants with down-regulated 4CL can result in enhanced disease resistance, and in particular, with increased fungal pathogen resistance. In particular, greenhouse transgenic aspen plants may show a disease resistance to fungi such as those which induce leaf-blight disease.

I. Promoters

Two distinct genes encoding 4CL and their promoters were cloned. The promoter of Pt4CL1 can drive gene expression specifically in xylem tissue and the promoter for Pt4CL2 confers gene expression exclusively in the epidermal tissues. These promoters can be used to manipulate gene expression to engineer traits of interest in specific tissues of target plants. The significance of the promoters is the application of the xylem-specific promoter to direct the expression of any relevant genes specifically in the xylem for engineering lignin content, lignin structure, enhanced growth, cellulose content, other value-added wood qualities, and the like. The importance of the epidermis-specific promoter is its ability to drive the expression of any relevant genes specifically in epidermal tissues for engineering disease-, UV light-, cold-, heat-, drought-, and other stress resistance traits in plants.

Specifically, the promoters of the Pt4CL1 and Pt4CL2 were isolated as follows. An aspen genomic library was screened with Pt4CL1 cDNA and Pt4CL2 partial cDNA fragment to isolate genomic clones of Pt4CL1 and Pt4CL2. Eleven and seven positive genomic clones were identified for Pt4CL1 and Pt4CL2 gene, respectively. Among 11 positive clones for Pt4CL1,

Pt4CL1g-4 contained a full length coding sequence and at least 2 kb of 5' flanking regions. The restriction map of Pt4CL1g-4 is set forth at Fig. 3.

With respect to Pt4CL2, restriction map analysis was performed on λ DNA of positive genomic clone Pt4CL2g-11 which contains a full length
5 coding sequence and about 1.2 kb of 5' flanking region. The restriction map of Pt4CL2g-11 is set forth at Fig. 4.

Approximately a 2.3 kb 5' flanking region of Pt4CL1 was digested from Pt4CL1g-4 using *Xba* I and *Sac* I sites and cloned into pGEM7Z *Xba* I and *Sac* I sites. The subcloned Pt4CL1 promoter was named p7Z-4XS and the restriction
10 map of P7Z-4XS is set forth at Fig 5. The 5' unilateral deletion of p7Z-4XS was generated for DNA sequencing by exonuclease III/S1 nuclease digestion using Erase-a-Base System (Promega, Madison, WI). The deletion series was sequenced using a primer on pGEM7Z vector.

A 1.5 kb *Hind* III and *Eco*R I fragment containing a 1.2 kb 5' flanking
15 region of Pt4CL2 and 0.3 kb coding region of Pt4CL2g-11 was subcloned in pBluescript II SK+ *Hind* III and *Eco*R I sites. The restriction map of the resulting clone, pSK-11HE, was determined by digesting the plasmid with several restriction enzymes, as in set forth at Fig. 6. In order to determine the sequence of the Pt4CL2 promoter, pSK-11HE was further digested into small
20 fragments according to the restriction map and subcloned into vectors with suitable cloning sites. The DNA sequence was determined using M13 universal primer and reverse primer on the vector.

The DNA sequences of the two promoters was determined and analyzed using Δ Taq cycle sequencing Kit (USB, Cleveland, OH), and GENETYX-MAC
25 7.3 sequence analysis software from Software Development Co., Ltd. The nucleotide sequence of promoter region of Pt4CL1 is set forth as SEQ ID NO:5 and the nucleotide sequence of the promoter region of Pt4CL2 is set forth as SEQ ID NO:6. The sequence of the promoter regions of Pt4CL1p and Pt4CL2p is available from Genbank, Accession Nos. AF041051 and AF041052,
30 respectively.

The insignificant sequence similarity between the 5'- and 3'-noncoding regions of these two genes and their distinct exon-intron organizations (four

introns in Pt4CL1 and five in Pt4CL2) further substantiate their functional and perhaps evolutionary divergency. Striking differences also were observed in the promoter sequences of these two genes. Three cis-acting elements, box P (CCTTTCACCAACCCCC; SEQ ID NO:15), box A (CCGTTC; SEQ ID

5 NO:16), and box L (TCTCACCAACC; SEQ ID NO:17), previously shown to be consensus in all known plant phenylalanine ammonialyase (PAL) and 4CL gene promoters (Hahlbrook et al., Proc. Natl. Acad. Sci. USA, 92, 4150 (1995); Logemann et al., Proc. Natl. Acad. Sci. USA, 92, 5905 (1995)), were identified within the 1kb 5' flanking sequence of Pt4CL1 (GenBank Accession No. AF041051). However, none of these boxes could be found within the analyzed 1.2 kb 5' flanking region of Pt4CL2 (GenBank Accession No. AF041052), suggesting that promoter differences between Pt4CL1 and Pt4CL2 genes could be responsible for the strikingly different patterns of tissue-specific expression of these genes, as observed in Northern analysis.

15 Tissue-specific expression can be achieved by fusing the promoters of Pt4CL1 or Pt4CL2 to a gene, e.g., an open reading frame of interest and transferred to a plant species via *Agrobacterium*. For the sake of example, the promoters of Pt4CL1 and Pt4CL2 were fused to a GUS reporter gene as detailed below. However, it should be noted that genes other than the GUS reporter gene 20 can be fused to these promoters for tissue specific expression.

In order to construct Pt4CL1 promoter-GUS binary vector, a 1 kb fragment covering 5'-flanking region and 17 bp coding region of Pt4CL1 was subcloned into pGEM7Z *Sph* I and *Eco*R I sites for constructing promoter-GUS binary vector. In this 1 kb DNA fragment, it is found that one *Xho* I site is 25 located at 486 bases upstream to the translation start site and the *Eco*R I site is located at 17 bases downstream the translation start site. This 0.6 kb fragment was subcloned into pGEM7Z *Xho* I and *Eco*R I sites and used as a template in PCR amplification.

In order to construct a promoter-GUS transcriptional fusion, a *Bam*H I 30 site was introduced in front of the translation start site of Pt4CL1 by PCR. PCR amplification was performed using p7Z-4XE as the template, M13 universal primer on pGEM7Z vector as 5' end primer and Pt4CL1p-1 primer containing a

*Bam*H I site at the end is complementary to a sequence upstream of the translation start site. The reaction was carried out in 100 µl reaction mix containing 1x *pfu* reaction buffer, 200 µl each dNTPs, 100 µM each primer and 5 units of *pfu*. The PCR reaction mixture was denatured at 94°C for 5 minutes
5 followed by 30 cycles of 94°C (1 minute), 55°C (1 minute), 72°C (1 minute, 30 seconds) and was ended with a 5 minute extension at 72°C.

The amplified 0.6 kb fragment was cloned and sequenced to confirm the sequence. The engineered 0.6 kb fragment was ligated to p7Z-4SE which was digested with *Xho* I and *Bam*H I. In order to incorporate a *Hind* III site in the 5'
10 end of Pt4CL1 promoter, the 1 kb *Sph* I-*Bam*H I PtCCL1 promoter region was the cloned into pNoTA (5 prime → 3 prime Inc., Boulder, CO) *Sph* I and *Bam*H I site. The 1 kb Pt4CL1 promoter was then released from pNoTA vector with *Hind* III and *Bam*H digestion and subsequently transcriptionally fused to pBI101 *Hind* III and *Bam*H I sites in front of GUS. The resulting binary vector was
15 named Pt4CL1p-GUS and is set forth at Fig. 7.

In order to construct Pt4CL2 promoter-GUS binary vector, pSK-11HE was digested with *Sph* I and *Eco*R I to release 0.2 kb *Sph* I and *Eco*R I fragment. The 0.2 kb fragment was cloned into pGEM7Z *Sph* I and *Eco*R I sites. A primer, Pt4CL2p-3' (5'-CATCGGATCCTGAGATGGAAGGGAGTTTCT-3'; SEQ ID
20 NO:15) was designed to be complementary to a sequence upstream of the translation start site of Pt4CL2 and to incorporate *Bam*H I site at the end. Amplification was performed using p7Z11SE as a template, M13 universal primer as the 5' end primer and Pt4CL2p-3 as the 3' end primer. A PCR reaction was carried out and the amplified PCR product was cloned and
25 sequenced to check the fidelity of the PCR amplification. The 0.2 kb *Sph* I-*Bam*H I DNA fragment with correct sequence was fused to pSK-11HE linearized with *Sph* I and *Bam*H I. The resulting plasmid was named pSK-11HB. The promoter of PtCCL2 was then excised from pSK-11HB with *Hind* III and *Bam*H I and ligated to PBI101 and *Hind* III and *Bam*H I site to make Pt4CL2p-GUS
30 transcriptional fusion binary vector as shown in Fig. 8.

The Pt4CL1p-GUS and Pt4CL2p-GUS constructs were then mobilized into *Agrobacterium tumefaciens* strain C58/pMP90 by freeze and thaw method as explained previously.

Leaf disk transformation of tobacco with these two *Agrobacterium* constructs is conducted according to the method of Horsch R.B. (1988) Leaf Disk Transformation, Plant Molecular Biology Manual, A5:1-9.

To further investigate the regulation of the tissue-specific expression of Pt4CL1 and Pt4CL2 genes at the cellular level, their promoter activities were analyzed in transgenic tobacco plants by histochemical staining of GUS gene expression driven by a 1 kb Pt4CL1 and 1.2 kb Pt4CL2 promoter sequences, respectively. In Pt4CL1p-GUS transgenic plants, intense GUS staining was detected in lignifying xylem of stem. Strong GUS activity also was found localized to xylem of leaf mid-rib and of root. However, there was no GUS expression in leaf blade, stem epidermis, cortex, phloem and pith, and flower petal. These results are consistent with the evidence that Pt4CL1 gene expression is xylem- or lignifying tissue-specific, and with the observation that Pt4CL1 mRNA level is the highest in aspen secondary developing xylem. In striking contrast to the Pt4CL1 promoter activity, the Pt4CL2 promoter did not direct GUS expression in vascular and xylem tissues in the stem and the leaf of Pt4CL23p-GUS transgenic plants. Instead, it directed GUS expression in lignin-deficient epidermal cells of the stem (Figure 10C) and of the leaf, reflecting the association of Pt4CL2 with nonlignin-related phenylpropanoid biosynthesis in the plant's outer layers. In addition, GUS staining also was detected in Pt4CL2p-GUS transgenic plant's floral organs, such as stigma and petal, suggesting the likely relevance of Pt4CL2 in mediating the formation of flavonoids, which are known to be accumulated in these organs (Higuchi (1997, supra; Caldwell et al., *Physiol. Plant*, 58, 455 (1983); Shirley, *Trends in Plant Sci.*, 1, 377 (1996)).

The epidermis-specific Pt4CL2 promoter activity indicated that the in vivo Pt4CL2 mRNA message observed in aspen stem internodes could be caused by the signal derived from the epidermis RNA. Thus, the specific expression of

Pt4CL2 mRNA in epidermis further supports the biochemical functions of Pt4CL2 protein in the biosynthesis of nonlignin-related phenylpropanoids.

Therefore, the promoter fragments incorporated in Pt4CL1p-GUS and Pt4CL2p-GUS fusion genes must encompass the regulatory sequence elements
5 that are responsible for the contrasting tissue-specific expression between Pt4CL1 and Pt4CL2 genes in aspen. Thus, based on both in vivo gene expression and gene promoter activity analyses, it was concluded that the expression of Pt4CL1 and Pt4CL2 genes in aspen is compartmentalized.

These results demonstrate that in aspen two functionally distinct 4CLs
10 are uniquely compartmentalized by their gene regulatory systems for mediating differentially the biosynthesis of lignin and other phenylpropanoids that serve different physiological functions in aspen. Pt4CL1 is involved in channeling hydroxycinnamic acid derivatives to the synthesis of guaiacyl-syringyl lignin in xylem tissues. Pt4CL2 is associated with the biosynthesis of phenylpropanoids
15 other than lignin in epidermal cells in the stem and the leaf, suggesting its likely participation in disease-resistance or defense-related mechanisms in the plant's outer layers. Therefore, 4CL isoforms may have distinct roles in plant defense systems and in lignification in a tissue-specific manner. From a practical point of view, the tissue-specific Pt4CL1 and Pt4CL2 gene promoters may offer a
20 more defined control of future genetic engineering of traits in trees that must be confined to xylem or epidermal cells.

J. Cellulose Accumulation

Twenty-five transgenic aspen lines were generated in which Pt4CL1 expression was down-regulated to various degrees by antisense inhibition, using
25 a Pt4CL1 gene operatively linked to a duplicated enhancer CaMV 35S promoter (Datla et al., Plant Sci., 94, 139 (1993)). The effect of Pt4CL1 deficiency on woody tissue development was investigated in ten-month-old trees. Pt4CL1 messenger RNA was drastically reduced in four lines (Fig. 9A). These lines also exhibited more than a 90% reduction in xylem Pt4CL1 enzyme activity (Fig.
30 9B), and a 40 to 45% reduction in stem lignin (Fig. 9C). A more modest lignin reduction was found in those lines with less drastic repression of Pt4CL1 activity. The reduction in lignin content was restricted to woody xylem, as

shown by attenuated lignin autofluorescence in xylem but not in phloem fibers following UV-irradiation (Figs. 9D, E). Severe repression of other lignin biosynthetic pathway enzymes, such as COMT or CAD, had no effect on lignin quantity in transgenic aspen, hybrid poplar or a loblolly pine (*Pinus taeda*) mutant (Tsai et al., 1998; VanDoorsselaere et al., *Plant J.*, 8, 855 (1995); Bauchner et al., *Plant Physiol.*, 112, 1479 (1996)). Lignin structure, however, was significantly altered in these cases.

To investigate the effect of Pt4CL1 repression on lignin structure, milled wood lignins were isolated from the stem of a transgenic (line 6 with a 45% lignin reduction) and a control (using methods described in Bjorkman, *Nature*, 174, 1057 (1954); Chiang et al., *Holzforschung*, 44, 147 (1990); and Ralph et al., *JACS*, 116, 9448 (1994)) and then were analyzed by nuclear magnetic resonance (NMR) Examination of HSQC (heteronuclear single-quantum coherence) spectra (Fig. 10) and their HSQC-TOCSY (HSQC-total correlation spectroscopy) counterparts and HMQC (heteronuclear multiple-quantum correlation) indicated that these lignins are structurally similar, consistent with their comparable syringyl-to-guaiacyl ratios based on thioacidolysis of intact stem. The ratios for control and transgenic line 6 were 2.3 and 2.1, respectively. Thus, there appeared to be little disruption of the normal lignin structure as a result of reduced Pt4CL1 activity. It is clear from Figure 10 that β -aryl ethers (β -O-4) **10**, normally the most abundant (50 to 60%) linkage type in tree lignin (Adler et al., *Wood Sci. Technol.*, 11, 169 (1977)), predominate in both lignin samples. In both lignins, erythro-isomers are more prevalent than their threo-counterparts, typical of angiosperm lignin. Resinol (β - β) units (**12** Fig. 10), which largely results from coupling of sinapyl alcohol **9b** monomers and represent initial intermediates in lignin polymerization reactions in angiosperm trees, are well represented in both lignins. Traces of phenylcoumaran (β -5) units **11** and α - β -diaryl ethers **14** were detectable in each lignin. Absent from both lignins were condensed biphenyl units such as dibenzodioxocins **13** (Ralph et al., *supra*). Such units, formed from 5-5-homo-coupling of coniferyl alcohol **9a**, normally represent about 4% of the constituents in angiosperm lignin (Adler, *supra*).

Low levels of 4-coumaric **2** and ferulic **4** acids are sometimes detectable in angiosperm lignins. Therefore, it was determined whether the incorporation of these acids was affected by decreased Pt4CL1 activity. Long-range ^{13}C - ^1H -correlation (HMQC) NMR experiments revealed that these acids were absent

5 from both lignin samples. However, cell walls of transgenic stem tissue contained alkaline extractable 4-coumaric **2** and ferulic **4** acids at levels 11- and 5-fold higher, respectively, than the control. Alkaline hydrolysis of stem wood meal (pass 80-mesh) was performed at room temperature for 24 hr in 1 N NaOH (Hartley, J. Chromatogr., 54, 335 (1971)). The hydrolysates were neutralized,

10 extracted with ethyl acetate and concentrated. The concentrated products were derivatized with BSTFA and analyzed by GC-MS in SIM (selected ion monitoring) mode using a DB-5 column. 4-Coumaric acid **2** (TMS-derivative; m/z 308) content of control was 199 ± 13 nmol/g dry wood, and 2145 ± 93 nmol/g dry wood in transgenic line 6. Ferulic acid **4** (TMS-derivative: m/z 338)

15 contents in control and transgenic line 6 were 510 ± 9 and 2431 ± 120 nmol/g dry wood, respectively. No sinapic acid **6** (TMS-derivative: m/z 368) could be detected in control. However, a significant amount of sinapic acid, 2452 ± 119 nmol/g dry wood, was found in transgenic line 6.

Together, the lignin and cell wall analyses support a requirement for

20 activation by Pt4CL1 of these phenolic acids for their incorporation into lignin. The cell wall apparently serves as a sink for accumulating these acids when Pt4CL1 activity is reduced. As a result, lignin content was reduced in the transgenic line but lignin composition and structure were not significantly altered. The conservation of normal lignin composition and structure in the

25 transgenic aspen stands in sharp contrast to the marked changes of lignin composition and structure in other transgenic and mutant plants with altered lignin biosynthesis (Tsai et al., 1998; Van Doorselaere et al., 1995; Baucher et al., 1996; Elkind et al., Proc. Natl. Acad. Sci. USA, 87, 9057 (1990); Piquemal et al., Plant J., 13, 17 (1998); Sewalt et al., Plant Physiol., 115, 41 (1997); Kajita et

30 al., Plant Physiol., 114, 871 (1997); Lee et al., Plant Cell, 9, 1985 (1997); Dwivedi et al., Plant Mol. Biol., 26, 61 (1994); Ni et al., Transgenic Res., 3, 120 (1994); Atanassova et al., Plant J., 8, 465 (1995); Halpin et al., Plant J., 6, 339

(1994); Hibino et al., *Biosci. Biotech. Biochem.*, 59, 929 (1995)). The results are consistent with the supposition that 4CL modulates lignin accumulation in trees in a regulatory manner that does not result in disruption of lignin structure.

Lignin and polysaccharides are proposed to account for the remarkable
5 mechanical strength of woody tissues (White et al., *Nature*, 205, 818 (1965);
Atalla et al., *Science*, 227, 636 (1985); Houtman et al., *Plant Physiol.*, 107, 977
(1995); Taylor et al., *Plant J.*, 2, 959 (1992); Turner et al., *Plant Cell*, 9, 689
(1997)). In consideration of the possible effects of severe lignin reduction on
structural polysaccharide components, these components were examined in stem
10 wood tissue. While hemicellulose content remained essentially unchanged, the
transgenic lines had a 9 to 15% increase in glucan (Table 7), identified as β -
(1-4)-glucan, or cellulose, by methylation-based linkage analysis and enzymatic
hydrolysis. Lignin content was determined as the sum of Klason and acid-
soluble lignins which represent the absolute quantity of lignin (Chiang et al.,
15 *Holzforschung*, 44, 147 (1990)). Cellulose and hemicelluloses contents were
determined based on the total sugars after acid hydrolysis of these
polysaccharides in stem woody tissue (Chiang et al., *Wood Sci. Technol.*, 17,
217 (1983); Pettersen et al., *J. Wood Chem. Technol.*, 11, 495 (1991)). Wood
meal (pass 80-mesh) was vacuum-dried at 45°C and hydrolyzed with H₂SO₄.
20 Sugar contents of the hydrolysates were determined by anion exchange high
performance liquid chromatography using pulsed amperometric detection and
used for quantifying glucan and other polysaccharides (hemicelluloses) (Davis, J.
Wood Chem. Technol., 18, 235 (1998)).

The dried wood meal was also used for methylation analysis of the
25 glucan in wood. Both the Hakomori (*J. Biochem. Tokyo*, 55, 205 (1964)) and
NaOH/CH₃I (Ciucanu et al., *Carbohydr. Res.*, 131, 209 (1984)) methylation
procedures were followed. Methylated samples were hydrolyzed in 2M TFA at
121°C for 2 hr, reduced with sodium borodeuteride, and acetylated using acetic
anhydride at 120°C for 3 hr. The derivatized samples were analyzed by GC-MS
30 using a Sp2330 Supelco column. The methylation revealed that the glucose
residues are mainly derived from 1-4 glucan for both control and transgenic

lines. Enzymatic hydrolysis of stem woody tissue further confirmed that the glucans in both control and transgenic lines are β -(1 \rightarrow 4)-glucan, or cellulose.

Thus, (1 \rightarrow 3)-linked glucan (callose), reportedly deposited in plant cell walls as a result of perturbed secondary metabolism (Schmelzer et al., Plant Cell, 1, 993 (1989)), was not detected in transgenic or control wood. Together, increased cellulose and decreased lignin content resulted in a cellulose-to-lignin ratio of 4 compared with 2 in control aspen (Table 7). The reason for the increased cellulose content is not clear. The absence of change in transcript levels of an aspen homolog of celA encoding a catalytic subunit of cellulose synthase (Arioli et al., Science, 279, 717 (1997)) argues against an increase in the rate of cellulose deposition due to altered transcriptional regulation in transgenic trees with reduced lignin content. The increase in cellulose content suggests that cross talk between lignin and cellulose biosynthetic pathways can nevertheless occur to ensure that cellulose biosynthesis becomes the preferred structural carbon sink when lignin biosynthesis is reduced. Because cellulose and lignin are the two components of wood most responsible for its rigidity, such cross talk could represent an adaptation to sustain mechanical strength in lignin deficient xylem.

The reduced lignin content in transgenic lines did not adversely affect tree growth and development. In fact, trees with down-regulated Pt4CL1 had thicker stems, longer internodes, and larger (frequently epinastic) leaves than controls (Figs. 11A and 11B). Scanning electron microscopy (SEM) revealed that the shape and size of stem xylem fiber and vessel cells were similar to those of controls (Figs. 11C-F). Therefore, the enhanced stem development in these transgenic lines was apparently due to increased proliferative activity during xylem development rather than to increased cell size. Root growth rates also increased in these lines, resulting in greater length (15-fold) and fresh weight gain (20-fold) than in controls over a 14-day period in ex vitro rooting experiments (Fig. 11G). Cell size distribution in the meristematic and elongation zones of root tips was similar in control and transgenic roots. As was the case in stem xylem, increased root growth rate of the transgenic was due to increased cell number. Leaf growth also increased in the transgenic lines resulting in 4- to

5-fold larger leaves than in controls (Fig. 11B). Mature leaf adaxial epidermal cells were measured in two of the transgenic lines and found to be at least twice as large as in control aspen. A more detailed analysis was conducted to determine whether the rate and/or the duration of cell expansion accounted for the increased cell size in mature leaves of transgenic aspen. Epidermal cell expansion stopped at leaf number 15 below the first emerging leaf in control plants, but epidermal cells as well as leaf area continued to expand at leaf number 28 in transgenics (Fig. 11H). Therefore, the prolonged expansion of epidermal cells contributed to increased leaf size in the transgenic aspen lines.

- 10 The promotive effects on growth and development in the transgenic trees was a surprising observation. Growth enhancement has not been reported in transgenic tobacco or *Arabidopsis* with downregulated PAL (phenylalanine ammonia lyase), CCR, C4H, 4CL, COMT, or CAD. In fact, stunted growth and collapsed cell walls occurred in some transgenic tobacco with altered lignin biosynthesis. Whether the growth responses between herbaceous and tree species differed due to altered lignin biosynthesis per se is not clear. In the case of aspen, lignin composition and structure were conserved, eliminating the possibility that altered lignin constituents promoted growth. In aspen trees, reduced expression of Pt4CL1 disrupted lignin biosynthesis downstream of the phenylpropanoid pathway and this increased the concentration of phenylpropanoid intermediates in cell walls. At the same time, enhanced cell division and cell expansion were observed in root tips and leaves. Whether the growth enhancement observed in the transgenic aspen is due to altered carbon distribution between primary/secondary metabolism or specifically due to changes in wall-bound moieties are two possibilities to consider. Histone gene(s) expression has been used as a marker to show that cell division decreases in suspension cells and young leaves of parsley following treatments of that divert carbon flow in to the phenylpropanoid pathway and away from primary metabolic pathways (Logemann et al., *Plant J.*, 8, 865 (1995)). There is also current interest in the organization and composition of cell wall constituents and their effects on cell expansion and plant growth. For these rationale,

phenylpropanoid flux as well as cell wall constituents would be of interest for investigating growth effects of lignin manipulation in trees.

The finding that cellulose content increases in transgenic aspen with disrupted lignin biosynthesis is unique; similar observations have not been reported in herbaceous plants (Turner et al., Plant Cell, 9, 689 (1997); Elkind et al., 1990; Piquemal et al., 1998)). Interesting to consider is the idea that in perennial woody plants, lignin and cellulose deposition in cell walls are regulated in a compensatory fashion such that decreased in one are compensated for by increases in the other for maintaining the cellular structural integrity. This compensatory deposition of lignin and cellulose is consistent with the manner of how trees regulate their lignin and cellulose quantities in the course of forming naturally occurring reaction wood for mechanical support. Compensatory regulation such as this would also provide metabolic flexibility during annual growth increments, perhaps key for the long term structural integrity of woody perennials like trees. Further study is required to determine whether such regulation of cellulose accumulation is sensitive to primary/secondary metabolism and to changes in cell wall constituents such as those observed in Pt4CL1 down-regulated aspen.

Overall, lignin limits the utilization of wood for fiber/material-, chemical-, and energy-production. Traditional breeding approaches have not led to trees with more desirable lignin/cellulose composition. However, genetic engineering appears to offer a strategy for manipulating such traits in trees, with the prospect of systemically regulating growth as reported here. The benefit of these engineered traits may also extend to forage crops in which lignin has been identified as the major barrier to their digestibility by ruminants.

Table 7. Lignin and cellulose contents in stem woody tissue of control and transgenic aspen. Data are the mean \pm SD of three independent experiments. Normalized values relative to control are shown in parentheses.

		Lignin Content	Cellulose Content	
5	Line	(% of dry wood weight)	(% of dry wood weight)	Cellulose-to-lignin ratio
	Control	21.62 \pm 0.30 (100)	44.23 \pm 0.43 (100)	2.0
	4	12.83 \pm 0.28 (60)	48.35 \pm 0.60 (109)	3.8
	5	13.02 \pm 0.28 (60)	49.74 \pm 0.45 (112)	3.7
	6	11.84 \pm 0.08 (55)	50.83 \pm 0.26 (115)	4.3
10	8	12.90 \pm 0.04 (60)	48.14 \pm 0.29 (109)	3.8

All publications and patents are incorporated by reference herein, as though individually incorporated by reference, as long as they are not inconsistent with the present disclosure. The invention is not limited to the exact details shown and described, for it should be understood that many variations and modifications may be made while remaining within the scope of the invention defined by the claims.

WHAT IS CLAIMED IS:

1. A method for altering the growth characteristics of a plant comprising the step of incorporating into the genome of the plant a recombinant
5 DNA molecule comprising a nucleotide sequence encoding 4-coumarate Co-enzyme A ligase such that when the nucleotide sequence is expressed in the plant, the growth of the plant is altered.
2. The method as set forth in claim 1 wherein the DNA molecule
10 comprises a homologous nucleotide sequence.
3. The method as set forth in claim 1 wherein the DNA molecule comprises a heterologous nucleotide sequence.
- 15 4. The method as set forth in claim 1 wherein the DNA molecule is incorporated into the genome of the plant by transformation using an Agrobacterium transfer vector.
5. The method as set forth in claim 1 wherein the DNA molecule
20 comprises the nucleotide sequence in antisense orientation.
6. The method as set forth in claim 1 wherein the DNA molecule comprises the nucleotide sequence in sense orientation.
- 25 7. The method as set forth in claim 6 wherein the DNA molecule is a cloned cDNA sequence of 4-coumarate Co-enzyme A ligase.
8. The method as set forth in claim 1 wherein the recombinant DNA molecule comprises the promoter sequence of CaMV35S.
30
9. The method as set forth in claim 1 wherein said altered growth is manifested as an increase in biomass.

10. A plant having its growth characteristic altered through the incorporation into the genome of the plant a recombinant DNA molecule comprising a nucleotide sequence encoding 4-coumarate Co-enzyme A ligase such that when the nucleotide sequence is expressed in the plant, the growth
5 characteristic of the plant is altered.

11. The plant as set forth in claim 10 wherein the DNA molecule comprises the nucleotide sequence in antisense orientation.

10 12. The plant as set forth in claim 10 wherein the DNA molecule comprises the nucleotide sequence in sense orientation.

13. The plant as set forth in claim 10 wherein the DNA is incorporated into the genome of the plant by transformation using an
15 Agrobacterium transfer vector.

14. The plant as set forth in claim 10 wherein the DNA molecule is a cloned cDNA sequence encoding 4-coumarate Co-enzyme A ligase.

20 15. The plant as set forth in claim 10 wherein the DNA molecule comprises the promoter of CaMV35S.

16. The plant as set forth in claim 10 wherein said altered growth is manifested as an increase in plant biomass.

25

17. The plant as set forth in claim 10 which is a tree.

18. A method for altering a characteristic of a plant comprising the step of genetically down regulating the enzyme 4-coumarate Co-enzyme A
30 ligase, wherein the characteristic is selected from the group consisting of accelerated growth, reduced lignin content, altered lignin structure, increased disease resistance and increased cellulose content.

19. The method of claim 18 wherein the plant is genetically down regulated through incorporation into the genome of the plant a recombinant DNA molecule comprising a nucleotide sequence encoding 4-coumarate Co-enzyme A ligase in antisense orientation.

5

20. The method as set forth in claim 18 wherein the recombinant DNA molecule is incorporated into the genome of the plant by transformation using an Agrobacterium transfer vector.

10

21. The method as set forth in claim 19 wherein the recombinant DNA molecule comprises a homologous nucleotide sequence that is incorporated into the genome of the plant.

15

22. The method as set forth in claim 18 wherein the nucleotide sequence is a cloned cDNA sequence encoding 4-coumarate Co-enzyme A ligase.

20

23. The method as set forth in claim 18 wherein the recombinant DNA molecule comprises a promoter of CaMV35S.

25

24. A plant having a characteristic altered by genetically down regulating the enzyme 4-coumarate Co-enzyme A ligase, wherein the characteristic is selected from the group consisting of accelerated growth, reduced lignin content, altered lignin structure, increased disease resistance and increased cellulose content.

30

25. The plant of claim 24 wherein the plant is genetically down regulated through incorporation into the genome of the plant a recombinant DNA molecule comprising a homologous nucleotide sequence encoding 4-coumarate Co-enzyme A ligase in the antisense orientation.

26. The plant of claim 24 wherein the recombinant DNA molecule is incorporated into the genome of the plant by transformation using an *Agrobacterium* transfer vector.

5 27. The plant of claim 24 wherein the nucleotide sequence is a cloned cDNA sequence of 4-coumarate Co-enzyme A ligase.

28. The plant of claim 24 wherein the recombinant DNA molecule comprises a promoter of CaMV35S.

10

29. An isolated and purified DNA molecule comprising a DNA segment comprising a transcriptional regulatory region of a plant 4-coumarate Co-enzyme A ligase gene.

15 30. The isolated and purified DNA molecule of claim 29 in which the DNA segment is from aspen.

31. The isolated and purified DNA molecule of claim 29 wherein the DNA segment directs expression of a linked sequence to the xylem of a plant.

20

32. The isolated and purified DNA molecule of claim 29 wherein the DNA segment directs expression of a linked sequence to the epidermal tissue of a plant.

25 33. A method of imparting disease resistance to a plant tissue comprising:

- (a) introducing an expression cassette comprising a recombinant DNA molecule comprising a nucleotide sequence encoding a 4-coumarate Co-enzyme A ligase operably linked to a promoter functional in a plant cell into cells of a plant;
- 30 (b) regenerating said plant cells to provide a transgenic plant; and

- (c) expressing the recombinant DNA molecule in the cells of the transgenic plant in an amount effective to render the plant resistant to disease.

5 34. The method according to claim 33 wherein the disease is caused by a fungus.

 35. The method according to claim 33 wherein the nucleotide sequence is in the antisense orientation.

10

 36. A transgenic plant prepared by the method of claim 33.

 37. A transgenic seed of the transgenic plant of claim 33.

15

 38. A transgenic plant, which plant is substantially resistant to disease, comprising:

- (a) a native 4-coumarate Co-enzyme A ligase gene, and
 (b) a recombinant DNA molecule comprising a nucleotide sequence encoding 4-coumarate Co-enzyme A ligase operably linked to a promoter functional in a plant wherein the recombinant DNA molecule is expressed in an amount effective to confer resistance to the transgenic plant.

20

 39. A method for altering the lignin content in a plant comprising:

25

 (a) introducing an expression cassette comprising a recombinant DNA molecule encoding a 4-coumarate Co-enzyme A ligase operably linked to a promoter functional in a plant cell into the cells of a plant;

 (b) regenerating said plant cells to provide a transgenic plant; and

 (c) expressing the recombinant DNA molecule in the cells of the

30

transgenic plant in an amount effective to alter the lignin content in the plant cells.

40. A transgenic plant having an altered lignin content in the plant cells comprising: a recombinant DNA molecule comprising a nucleotide sequence encoding a plant 4-coumarate Co-enzyme A ligase operably linked to a promoter so that the recombinant DNA molecule is expressed in an amount
5 effective to alter the lignin content of the plant.

41. A method for altering the cellulose content in a plant comprising:
(a) introducing an expression cassette comprising a recombinant DNA molecule comprising a nucleotide sequence encoding a 4-coumarate Co-
10 enzyme A ligase operably linked to a promoter functional in a plant cell into the cells of a plant;
(b) regenerating said plant cells to provide a transgenic plant; and
(c) expressing the recombinant DNA molecule in the cells of the transgenic plant in an amount effective to alter the cellulose content in the plant.

15 42. A transgenic plant having an altered cellulose content in the plant cells comprising: a recombinant DNA molecule comprising a recombinant DNA molecule sequence encoding a plant 4-coumarate Co-enzyme A ligase operably linked to a promoter so that the recombinant DNA molecule is expressed in an
20 amount effective to alter the cellulose content of the plants.

43. A method for altering the lignin structure in a plant comprising:
(a) introducing an expression cassette comprising a recombinant DNA molecule comprising a recombinant DNA nucleotide sequence encoding a
25 4-coumarate Co-enzyme A ligase operably linked to a promoter functional in a plant cell into the cells of a plant;
(b) regenerating said plant cells to provide a transgenic plant; and
(c) expressing the recombinant DNA molecule in the cells of the transgenic plant in an amount effective to alter the lignin structure in the plants.

30 44. A transformed plant having an altered lignin structure comprising: a recombinant DNA molecule comprising a nucleotide sequence encoding a plant 4-coumarate Co-enzyme A ligase operably linked to a promoter so that the

recombinant DNA molecule is expressed in an amount effective to alter the lignin structure of the plant.

45. An expression cassette comprising a transcriptional control region
5 of a 4-coumarate Co-enzyme A ligase gene operably linked to a DNA segment comprising an open reading frame.

46. A method of expressing a DNA segment in the xylem of a plant, comprising:

- 10 (a) introducing an expression cassette comprising a transcriptional control region of a 4-coumarate Co-enzyme A ligase gene operably linked to a DNA segment into cells of a plant;
- (b) regenerating the plant cells to provide a transgenic plant; and
- (c) expressing the DNA segment in the xylem of a plant.

15 47. A method of expressing a DNA segment in the epidermal tissue of a plant, comprising:

- (a) introducing an expression cassette comprising a transcriptional control region of a 4-coumarate Co-enzyme A ligase gene purple
- 20 operably linked to a DNA segment into cells of a plant;
- (b) regenerating the plant cells to provide a transgenic plant; and
- (c) expressing the DNA segment in the epidermal tissue of a plant.

48. The method of claim 46 wherein the transgenic plant has altered
25 lignin content, lignin structure, cellulose content or wood quality relative to the corresponding non-transgenic plant.

49. The plant of claim 38 which has altered levels of
phenylpropanoids or other secondary metabolites relative to the corresponding
30 non-transgenic plant.

50. The method of claim 1 wherein the plant has enhanced root growth.

51. The plant of claim 10 wherein the plant has enhanced root growth.

52. The method of claim 1 wherein the plant has enhanced root
5 development.

53. The plant of claim 10 wherein the plant has enhanced root development.

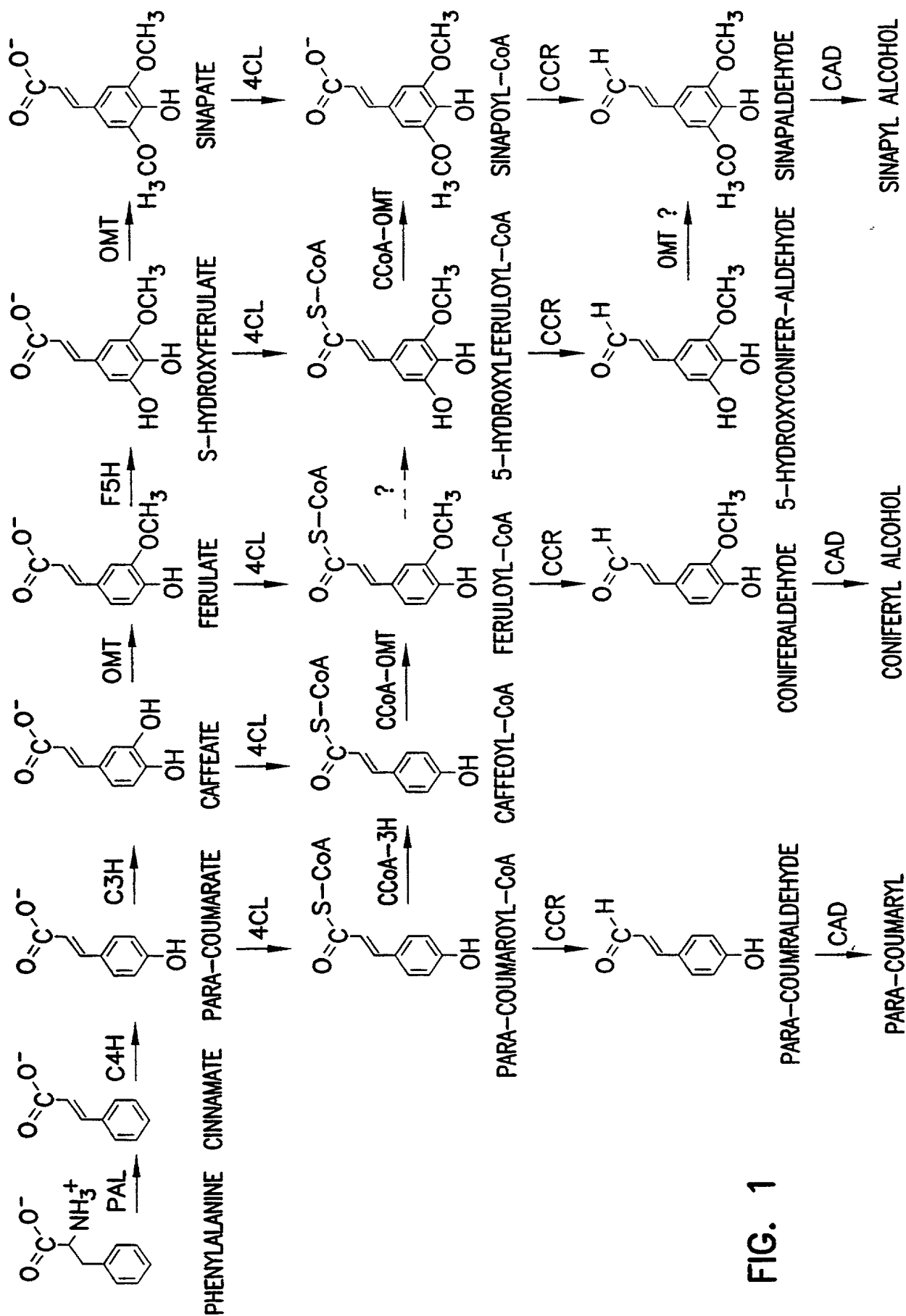


FIG. 1

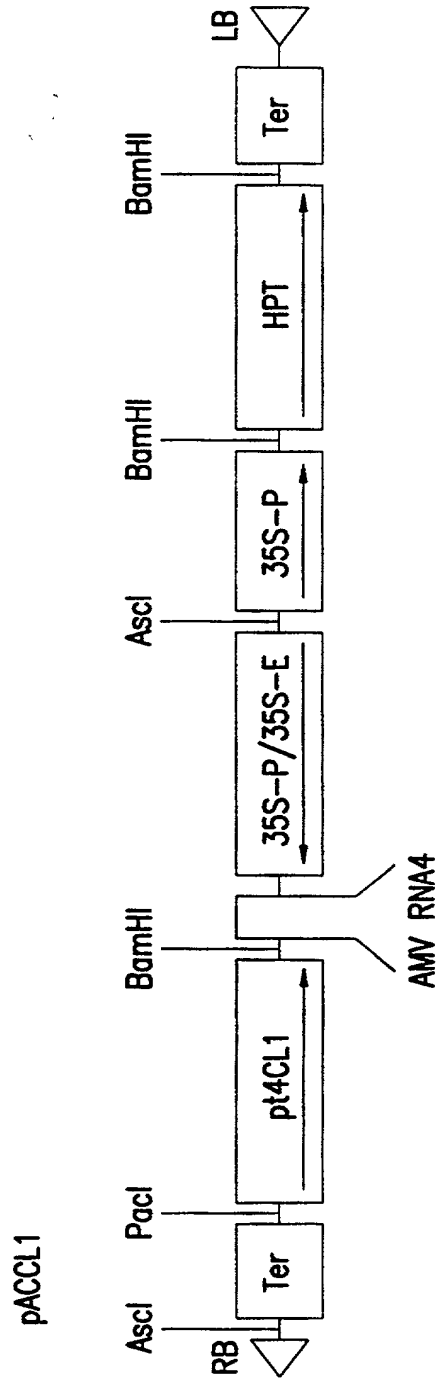


FIG. 2



Pt4CL2g-11

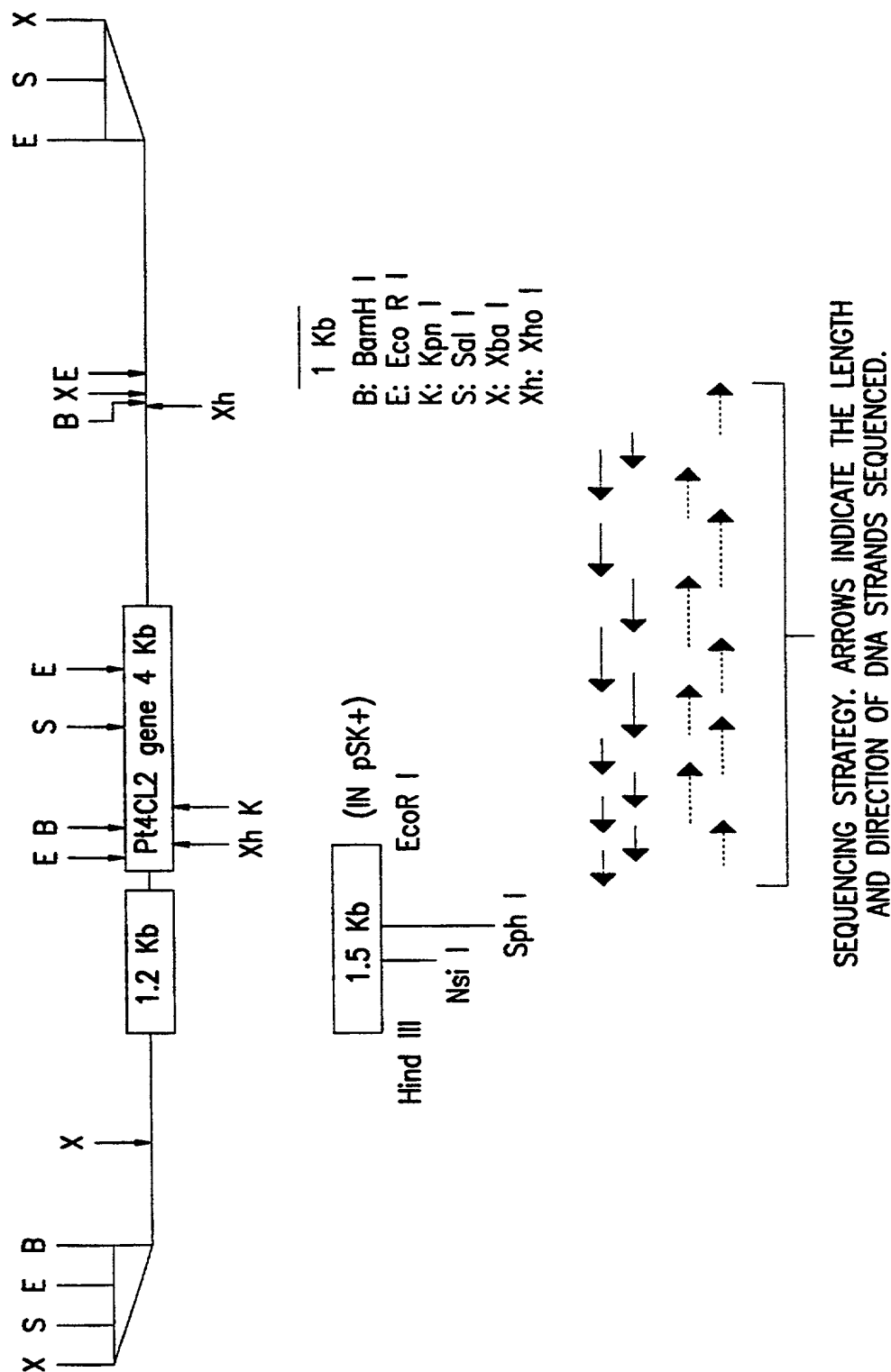


FIG. 4

p7Z-4XS

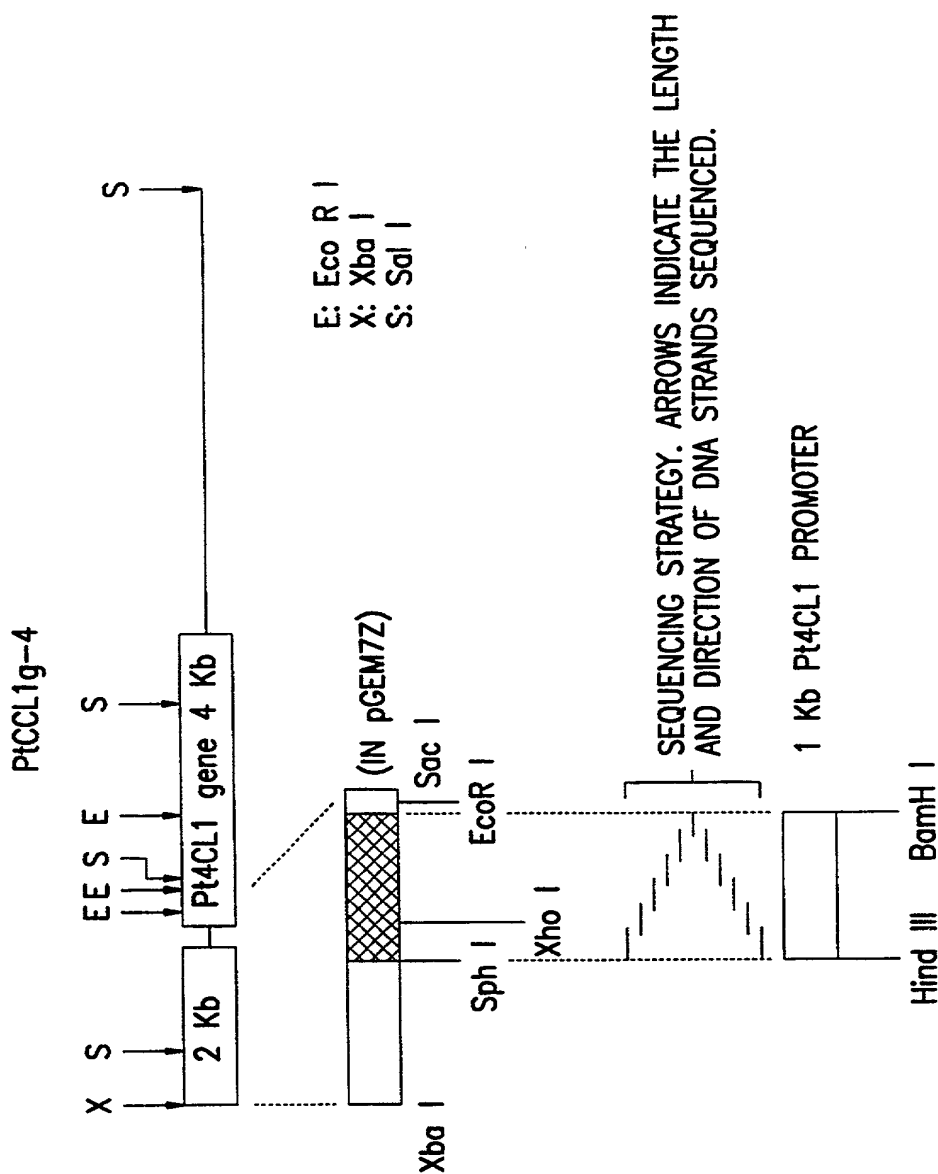


FIG. 5

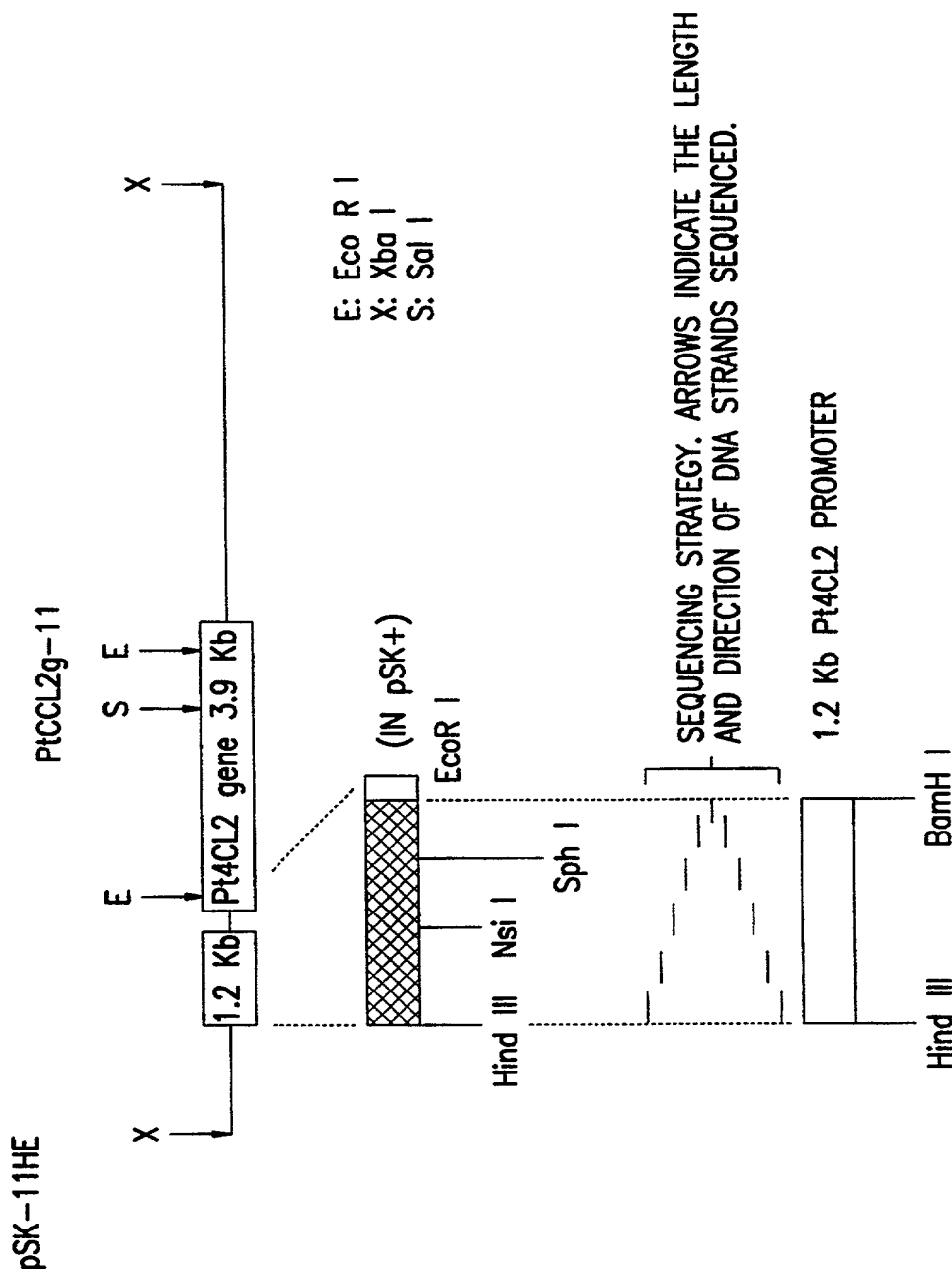


FIG. 6

Pt4CL1p-GUS

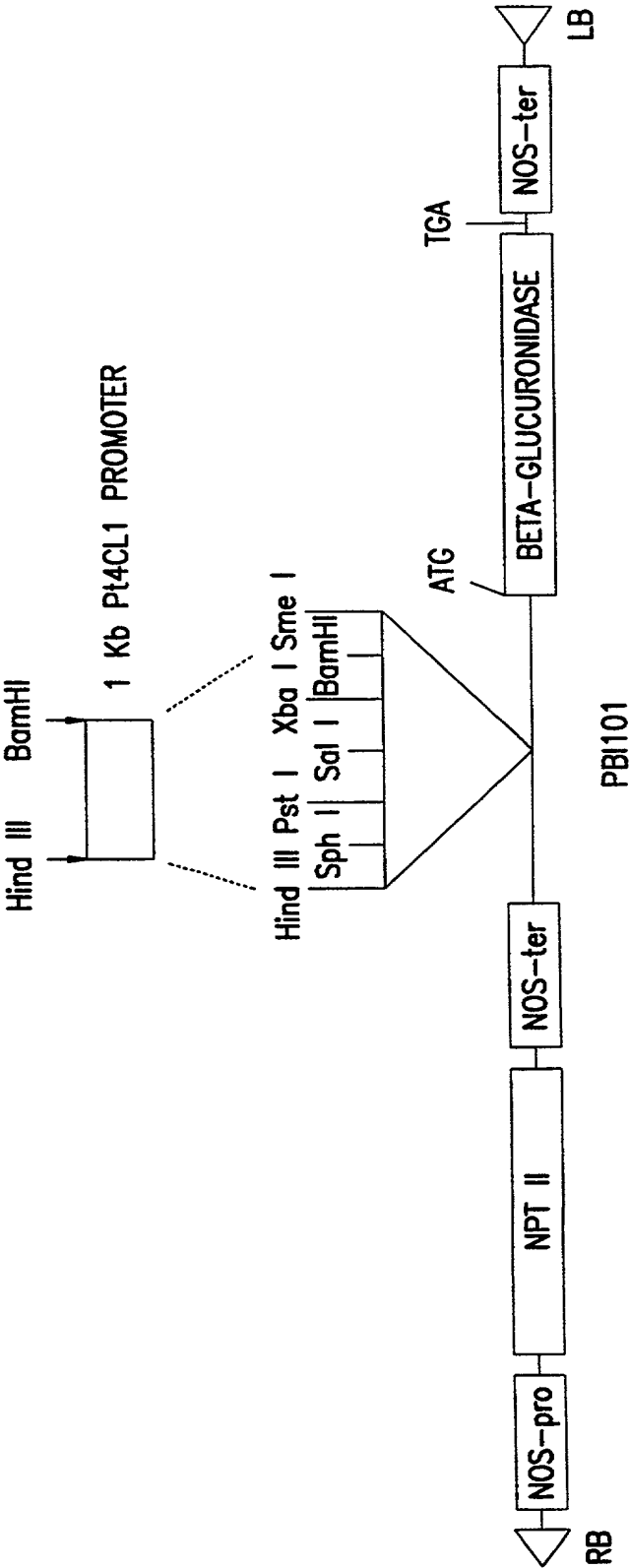


FIG. 7

Pt4CL2p-GUS

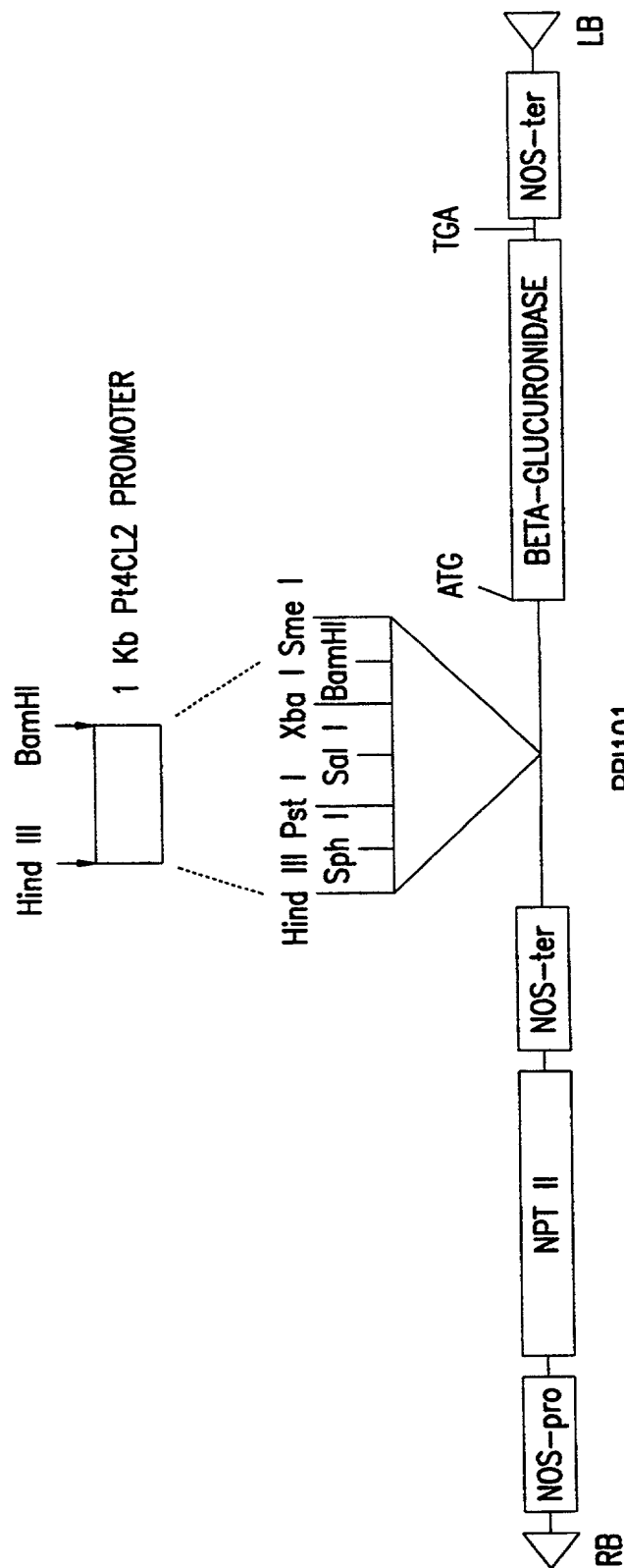


FIG. 8

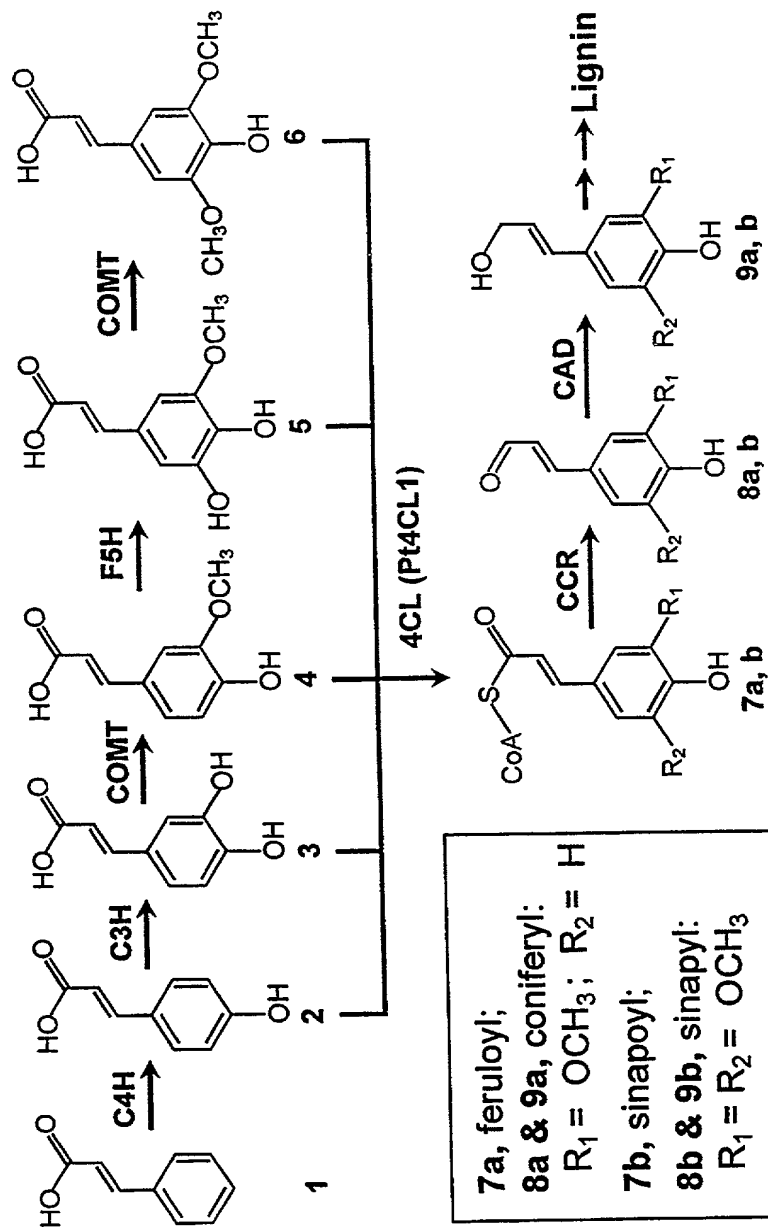


FIG. 9

C 9 3 4 5 6 8

FIG. 10A

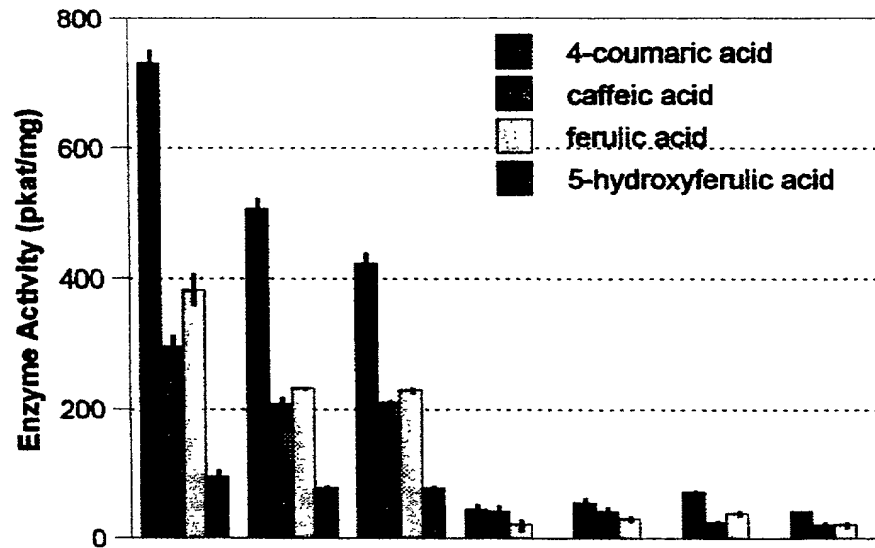


FIG. 10B

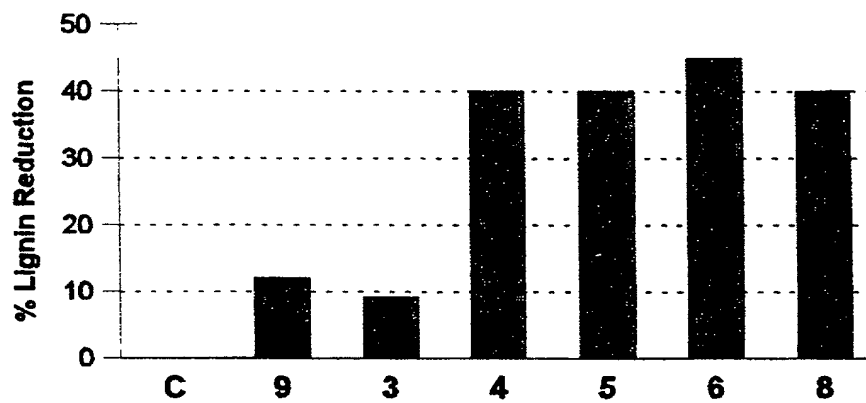


FIG. 10C

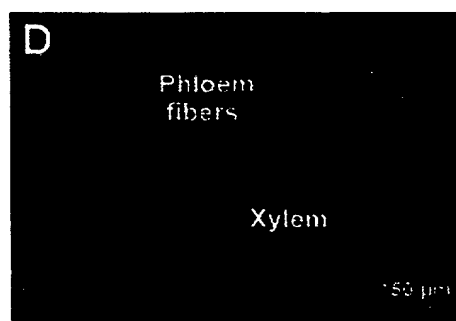


FIG. 10D

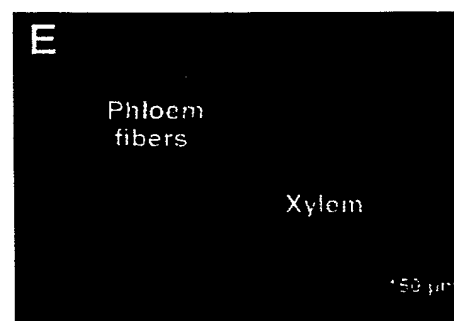


FIG. 10E

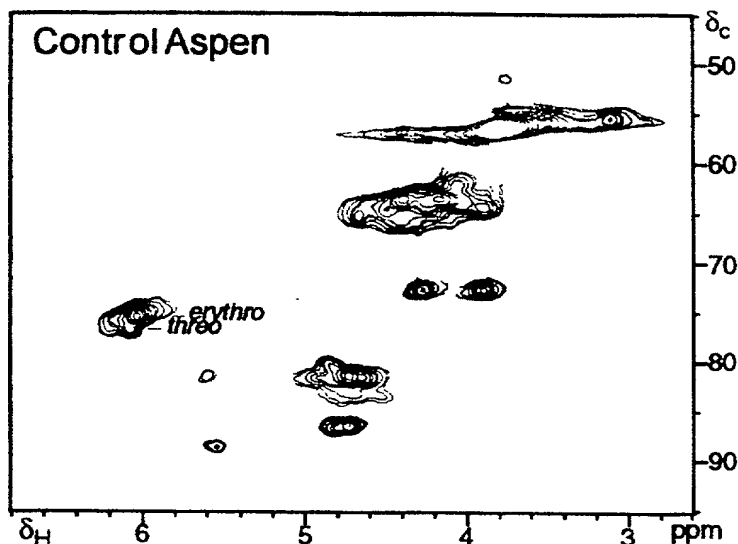


FIG. 11A

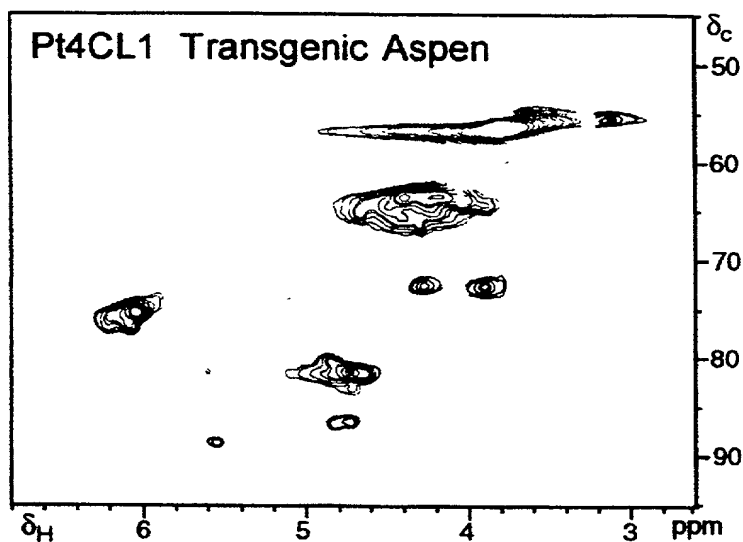


FIG. 11B

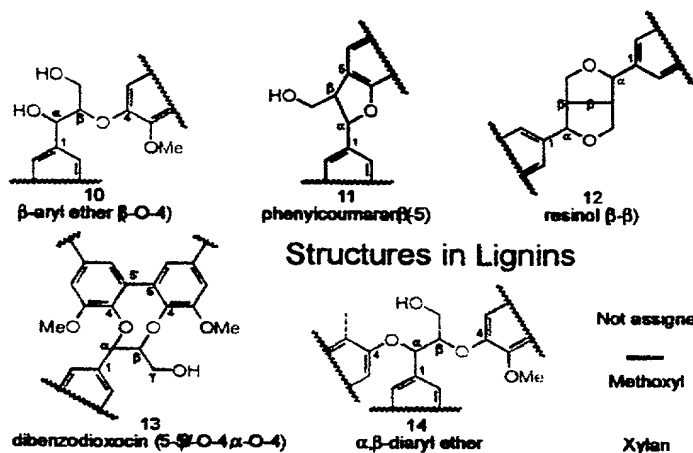
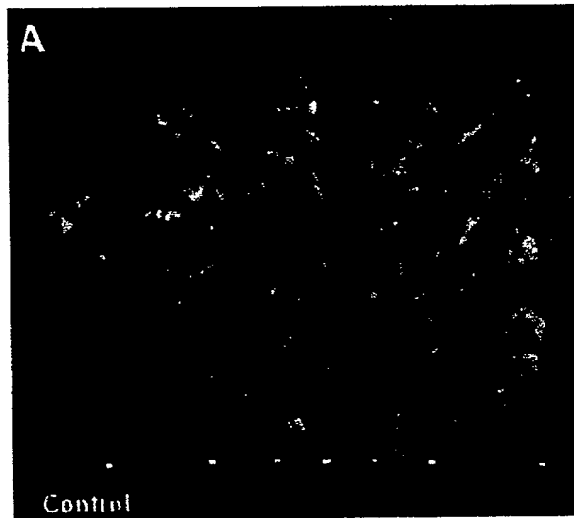
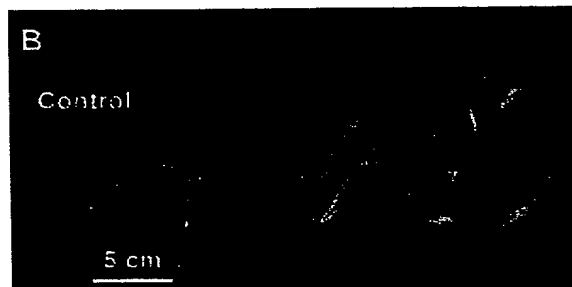
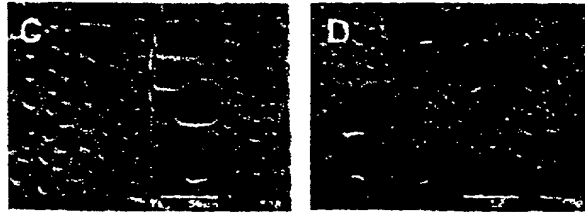
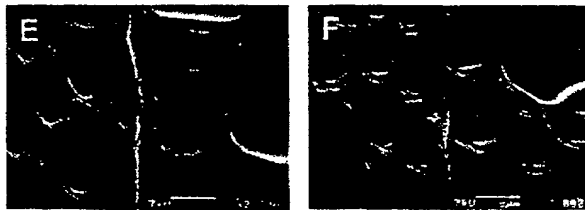
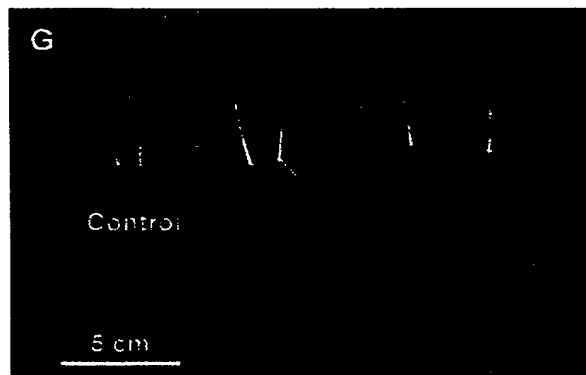


FIG. 11C

**FIG. 12A****FIG. 12B**

**FIG. 12C FIG. 12D****FIG. 12E FIG. 12F****FIG. 12G**

14/14

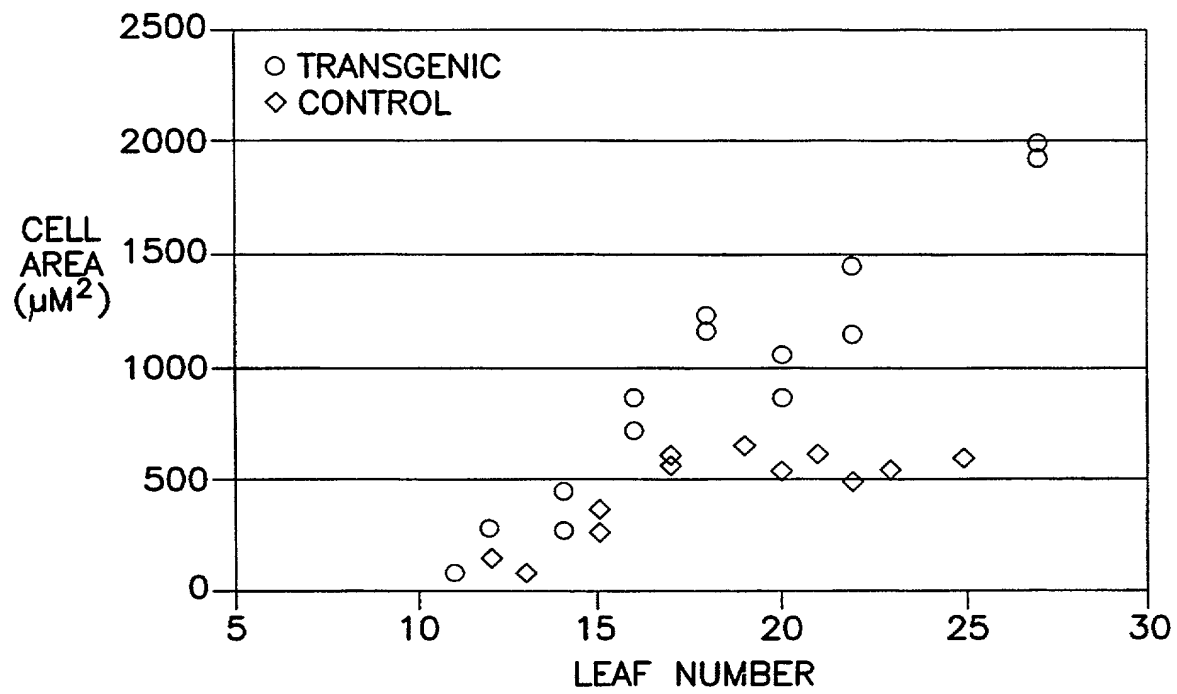


FIG. 12H

Declaration and Power of Attorney For Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled GENETIC ENGINEERING OF LIGNIN BIOSYNTHESIS IN PLANTS (Attorney Docket No. 66040-9651-01), the specification of which was filed with my authority, on May 3, 2000 as Application Serial No. 09/530,663.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims referred to above.

I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

And I hereby appoint Joseph A. Gemignani (Reg. No. 19,482), Christopher B. Austin (Reg. No. 41,592), Robert E. Clemency (Reg. No. 19,287), David L. De Bruin (Reg. No. 35,489), Gerald L. Fellows (Reg. No. 36,133), Randall W. Fieldhack (Reg. No. 43,611), Gregory J. Hartwig (Reg. No. P-46,761), Daniel S. Jones (Reg. No. 42,697), Richard L. Kaiser (Reg. No. 46,158), Timothy M. Kelley (Reg. No. 34,201), Casimir F. Laska (Reg. No. 30,862), Edward R. Lawson, Jr. (Reg. No. 41,931), Richard H. Marschall (Reg. No. 39,290), Thomas A. Miller (Reg. No. 36,871), Kevin P. Moran (Reg. No. 37,193), Andrew R. Pèret (Reg. No. 41,246), David R. Price (Reg. No. 31,557), Thomas S. Reynolds II (Reg. No. 45,262), David B. Smith (Reg. No. 27,595), Derek C. Stettner (Reg. No. 37,945), Billie Jean Strandt (Reg. No. 36,940), Sheldon L. Wolfe (Reg. No. 43,996), Paul F. Donovan (Reg. No. 39,962), Jill A. Fahrlander (Reg. No. 42,518), Grady J. Frenchick (Reg. No. 29,018), Karen B. King (Reg. No. 41,898), Linda Blair Meier (Reg. No. 39,769), Teresa J. Welch (Reg. No. 33,049), Robert S. Beiser (Reg. No. 28,687), Witold A. Ziarno (Reg. No. 39,888), and each or any of them, my attorneys or agents, with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

TO: ADDRESS ALL COMMUNICATIONS IN OR PERTAINING TO THIS APPLICATION

Joseph A. Gemignani
Michael Best & Friedrich LLP
100 East Wisconsin Avenue
Milwaukee, Wisconsin 53202-4108

I hereby claim the benefit under Title 35, United States Code, §120 of the United States applications listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a)

which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.
08/969,046

Filing Date
12 November 1997 ✓

Status
Pending

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of the foreign application for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PCT/US98/24138 ✓
(Number)

Prior Foreign Application
PCT
(Country)

12 November 1998 ✓
(Day/Month/Year Filed)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00 Full name of first joint inventor: Vincent Lee C. Chiang ✓

Inventor's signature Vincent L. C. Chiang

Date: 7-7-00

Residence: Hancock, Michigan MI

Citizenship: United States of America ✓

Post Office Address: 1104 Birch Street

Hancock, MI 49930

2-00 Full name of second joint inventor: Chung-Jui Tsai ✓

Inventor's signature Chung-Jui Tsai

Date: 7-6-00

Residence: Hancock, Michigan MI

Citizenship: Taiwan ✓

Post Office Address: 1510 Anthony Street #34

Hancock, MI 49930

Full name of third joint inventor: Wen-Jing Hu

Inventor's signature _____

Date: _____

Residence: Houston, Texas

Citizenship: Taiwan

Post Office Address: 8299 Cambridge Drive #1003

Houston, TX 77054

Declaration and Power of Attorney For Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled GENETIC ENGINEERING OF LIGNIN BIOSYNTHESIS IN PLANTS (Attorney Docket No. 66040-9651-01), the specification of which was filed with my authority, on May 3, 2000 as Application Serial No. 09/530,663.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims referred to above.

I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

And I hereby appoint Joseph A. Gemignani (Reg. No. 19,482), Christopher B. Austin (Reg. No. 41,592), Robert E. Clemency (Reg. No. 19,287), David L. De Bruin (Reg. No. 35,489), Gerald L. Fellows (Reg. No. 36,133), Randall W. Fieldhack (Reg. No. 43,611), Gregory J. Hartwig (Reg. No. P-46,761), Daniel S. Jones (Reg. No. 42,697), Richard L. Kaiser (Reg. No. 46,158), Timothy M. Kelley (Reg. No. 34,201), Casimir F. Laska (Reg. No. 30,862), Edward R. Lawson, Jr. (Reg. No. 41,931), Richard H. Marschall (Reg. No. 39,290), Thomas A. Miller (Reg. No. 36,871), Kevin P. Moran (Reg. No. 37,193), Andrew R. Peret (Reg. No. 41,246), David R. Price (Reg. No. 31,557), Thomas S. Reynolds II (Reg. No. 45,262), David B. Smith (Reg. No. 27,595), Derek C. Stettner (Reg. No. 37,945), Billie Jean Strandt (Reg. No. 36,940), Sheldon L. Wolfe (Reg. No. 43,996), Paul F. Donovan (Reg. No. 39,962), Jill A. Fahrlander (Reg. No. 42,518), Grady J. Frenchick (Reg. No. 29,018), Karen B. King (Reg. No. 41,898), Linda Blair Meier (Reg. No. 39,769), Teresa J. Welch (Reg. No. 33,049), Robert S. Beiser (Reg. No. 28,687), Witold A. Ziarno (Reg. No. 39,888), and each or any of them, my attorneys or agents, with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

TO: ADDRESS ALL COMMUNICATIONS IN OR PERTAINING TO THIS APPLICATION

Joseph A. Gemignani
Michael Best & Friedrich LLP
100 East Wisconsin Avenue
Milwaukee, Wisconsin 53202-4108

I hereby claim the benefit under Title 35, United States Code, §120 of the United States applications listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a)

which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.
08/969,046

Filing Date
12 November 1997

Status
Pending

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of the foreign application for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PCT/US98/24138
(Number)

Prior Foreign Application
PCT
(Country)

12 November 1998
(Day/Month/Year Filed)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first joint inventor: Vincent Lee C. Chiang

Inventor's signature _____

Date: _____

Residence: Hancock, Michigan
Citizenship: United States of America
Post Office Address: 1104 Birch Street
Hancock, MI 49930

Full name of second joint inventor: Chung-Jui Tsai

Inventor's signature _____

Date: _____

Residence: Hancock, Michigan
Citizenship: Taiwan
Post Office Address: 1510 Anthony Street #34
Hancock, MI 49930

3-00 Full name of third joint inventor: Wen-Jing Hu ✓

Inventor's signature Wen-Jing Hu _____

Date: 7/6/2000

Residence: Houston, Texas TX
Citizenship: Taiwan
Post Office Address: 8299 Cambridge Drive #1003
Houston, TX 77054

SEQUENCE LISTING

<110> The Board of Control of
Michigan Technological University et al.

<120> GENETIC ENGINEERING OF LIGNIN BIOSYNTHESIS IN PLANTS

<130> 881.003WO1

<140>

<141> Herewith

<150> 08/969,046

<151> 1997-11-12

<160> 17

<170> FastSEQ for Windows Version 3.0

<210> 1

<211> 1915

<212> DNA

<213> Populus tremuloides Michx. (aspen)

<220>

<221> CDS

<222> (83)...(1687)

<400> 1

ccctcgcgaa actccgaaaa cagagagcac ctaaaaactca ccatctctcc ctctgcatct	60
ttagcccgcga atggacgccca ca atg aat cca caa gaa ttc atc ttt cgc tca	112
Met Asn Pro Gln Glu Phe Ile Phe Arg Ser	
1 5 10	
aaa tta cca gac atc tac atc ccg aaa aac ctt ccc ctg cat tca tac	160
Lys Leu Pro Asp Ile Tyr Ile Pro Lys Asn Leu Pro Leu His Ser Tyr	
15 20 25	
gtt ctt gag aac ttg tct aaa cat tca tca aaa cct tgc ctg ata aat	208
Val Leu Glu Asn Leu Ser Lys His Ser Ser Lys Pro Cys Leu Ile Asn	
30 35 40	
ggc gcg aat gga gat gtc tac acc tat gct gat gtt gag ctc aca gca	256
Gly Ala Asn Gly Asp Val Tyr Thr Tyr Ala Asp Val Glu Leu Thr Ala	
45 50 55	
aga aga gtt gct tct ggt ctg aac aag att ggt att caa caa ggt gac	304
Arg Arg Val Ala Ser Gly Leu Asn Lys Ile Gly Ile Gln Gln Gly Asp	
60 65 70	
gtg atc atg ctc ttc cta cca agt tca cct gaa ttc gtg ctt gct ttc	352
Val Ile Met Leu Phe Leu Pro Ser Ser Pro Glu Phe Val Leu Ala Phe	
75 80 85 90	
cta ggc gct tca cac aga ggt gcc atg atc act gct gcc aat cct ttc	400
Leu Gly Ala Ser His Arg Gly Ala Met Ile Thr Ala Ala Asn Pro Phe	
95 100 105	
tcc acc cct gca gag cta gca aaa cat gcc aag gcc tcg aga gca aag	448
Ser Thr Pro Ala Glu Leu Ala Lys His Ala Lys Ala Ser Arg Ala Lys	

Sequence Listing

110					115					120						
ctt	ctg	ata	aca	cag	gct	tgt	tac	tac	gag	aag	gtt	aaa	gat	ttt	gcc	496
Leu	Leu	Ile	Thr	Gln	Ala	Cys	Tyr	Tyr	Glu	Lys	Val	Lys	Asp	Phe	Ala	
		125					130					135				
cga	gaa	agt	gat	gtt	aag	gtc	atg	tgc	gtg	gac	tct	gcc	ccg	gac	ggg	544
Arg	Glu	Ser	Asp	Val	Lys	Val	Met	Cys	Val	Asp	Ser	Ala	Pro	Asp	Gly	
	140					145					150					
gct	tca	ctt	ttc	aga	gct	cac	aca	cag	gca	gac	gaa	aat	gaa	gtg	cct	592
Ala	Ser	Leu	Phe	Arg	Ala	His	Thr	Gln	Ala	Asp	Glu	Asn	Glu	Val	Pro	
155					160					165					170	
cag	gtc	gac	att	agt	cct	gat	gat	gtc	gta	gca	ttg	cct	tat	tca	tca	640
Gln	Val	Asp	Ile	Ser	Pro	Asp	Asp	Val	Val	Ala	Leu	Pro	Tyr	Ser	Ser	
				175					180					185		
ggg	act	aca	ggg	ttg	cca	aaa	ggg	gtc	atg	tta	acg	cac	aaa	ggg	cta	688
Gly	Thr	Thr	Gly	Leu	Pro	Lys	Gly	Val	Met	Leu	Thr	His	Lys	Gly	Leu	
			190					195					200			
ata	acc	agt	gtg	gct	caa	cag	gta	gat	gga	gac	aat	cct	aac	ctg	tat	736
Ile	Thr	Ser	Val	Ala	Gln	Gln	Val	Asp	Gly	Asp	Asn	Pro	Asn	Leu	Tyr	
		205					210					215				
ttt	cac	agt	gaa	gat	gtg	att	ctg	tgt	gtg	ctt	cct	atg	ttc	cat	atc	784
Phe	His	Ser	Glu	Asp	Val	Ile	Leu	Cys	Val	Leu	Pro	Met	Phe	His	Ile	
	220					225					230					
tat	gct	ctg	aat	tca	atg	atg	ctc	tgt	ggg	ctg	aga	gtt	ggg	gcc	tcg	832
Tyr	Ala	Leu	Asn	Ser	Met	Met	Leu	Cys	Gly	Leu	Arg	Val	Gly	Ala	Ser	
235					240					245					250	
att	ttg	ata	atg	cca	aag	ttt	gag	att	ggg	tct	ttg	ctg	gga	ttg	att	880
Ile	Leu	Ile	Met	Pro	Lys	Phe	Glu	Ile	Gly	Ser	Leu	Leu	Gly	Leu	Ile	
				255					260					265		
gag	aag	tac	aag	gta	tct	ata	gca	cca	gtt	gtt	cca	cct	gtg	atg	atg	928
Glu	Lys	Tyr	Lys	Val	Ser	Ile	Ala	Pro	Val	Val	Pro	Pro	Val	Met	Met	
			270					275					280			
gca	att	gct	aag	tca	cct	gat	ctt	gac	aag	cat	gac	ctg	tct	tct	ttg	976
Ala	Ile	Ala	Lys	Ser	Pro	Asp	Leu	Asp	Lys	His	Asp	Leu	Ser	Ser	Leu	
		285					290					295				
agg	atg	ata	aaa	tct	gga	ggg	gct	cca	ttg	ggc	aag	gaa	ctt	gaa	gat	1024
Arg	Met	Ile	Lys	Ser	Gly	Gly	Ala	Pro	Leu	Gly	Lys	Glu	Leu	Glu	Asp	
	300					305					310					
act	gtc	aga	gct	aag	ttt	cct	cag	gct	aga	ctt	ggg	cag	gga	tat	gga	1072
Thr	Val	Arg	Ala	Lys	Phe	Pro	Gln	Al								

gca gag atg aag att gtt gac cca gaa aca ggg gtc tct cta ccg agg 1216
Ala Glu Met Lys Ile Val Asp Pro Glu Thr Gly Val Ser Leu Pro Arg
365 370 375

aac cag cct ggt gag atc tgc atc cgg ggt gat cag atc atg aaa gga 1264
Asn Gln Pro Gly Glu Ile Cys Ile Arg Gly Asp Gln Ile Met Lys Gly
380 385 390

tat ctt aat gac ccc gag gca acc tca aga aca ata gac aaa gaa gga 1312
Tyr Leu Asn Asp Pro Glu Ala Thr Ser Arg Thr Ile Asp Lys Glu Gly
395 400 405 410

tgg ctg cac aca ggc gat atc ggc tac att gat gat gat gat gag ctt 1360
Trp Leu His Thr Gly Asp Ile Gly Tyr Ile Asp Asp Asp Asp Glu Leu
415 420 425

ttc atc gtt gac aga ttg aag gaa ttg atc aag tat aaa ggg ttt cag 1408
Phe Ile Val Asp Arg Leu Lys Glu Leu Ile Lys Tyr Lys Gly Phe Gln
430 435 440

gtt gct cct act gaa ctc gaa gct ttg tta ata gcc cat cca gag ata 1456
Val Ala Pro Thr Glu Leu Glu Ala Leu Leu Ile Ala His Pro Glu Ile
445 450 455

tcc gat gct gct gta gta gga ttg aaa gat gag gat gcg gga gaa gtt 1504
Ser Asp Ala Ala Val Val Gly Leu Lys Asp Glu Asp Ala Gly Glu Val
460 465 470

cct gtt gca ttt gta gtg aaa tca gaa aag tct cag gcc acc gaa gat 1552
Pro Val Ala Phe Val Val Lys Ser Glu Lys Ser Gln Ala Thr Glu Asp
475 480 485 490

gaa att aag cag tat att tca aaa cag gtg atc ttc tac aag aga ata 1600
Glu Ile Lys Gln Tyr Ile Ser Lys Gln Val Ile Phe Tyr Lys Arg Ile
495 500 505

aaa cga gtt ttc ttc att gaa gca att ccc aag gca cca tca ggc aag 1648
Lys Arg Val Phe Phe Ile Glu Ala Ile Pro Lys Ala Pro Ser Gly Lys
510 515 520

atc ctg agg aag aat ctg aaa gag aag ttg cca ggc ata taactgaaga 1697
Ile Leu Arg Lys Asn Leu Lys Glu Lys Leu Pro Gly Ile
525 530 535

tggtactgaa catttaaccc tctgtcttat ttctttaata cttgcgaatc attgtagtgt 1757
tgaaccaagc atgcttgaa aagacacgta cccaacgtaa gacagttact gttcctagta 1817
tacaagctct ttaatgttcg ttttgaactt gggaaaacat aagttctcct gtcgccatat 1877
ggagtaattc aattgaatat tttggtttct ttaatgat 1915

<210> 2
<211> 535
<212> PRT
<213> Populus tremuloides Michx. (aspen)

<400> 2
Met Asn Pro Gln Glu Phe Ile Phe Arg Ser Lys Leu Pro Asp Ile Tyr
1 5 10 15
Ile Pro Lys Asn Leu Pro Leu His Ser Tyr Val Leu Glu Asn Leu Ser
20 25 30

Sequence

[illegible]

515 520 525
 Lys Glu Lys Leu Pro Gly Ile
 530 535

<210> 3
 <211> 1710
 <212> DNA
 <213> Populus tremuloides Michx. (aspen)

<220>
 <221> CDS
 <222> (1)...(1710)

<400> 3
 atg atg tcc gtg gcc acg gtt gag ccc ccg aaa ccg gaa ctc tcc cct 48
 Met Met Ser Val Ala Thr Val Glu Pro Pro Lys Pro Glu Leu Ser Pro
 1 5 10 15
 cca caa aac caa aac gca cca tcc tct cat gaa act gat cac att ttc 96
 Pro Gln Asn Gln Asn Ala Pro Ser Ser His Glu Thr Asp His Ile Phe
 20 25 30
 aga tca aaa cta cca gac ata acc atc tcg aac gac ctc cct ctg cac 144
 Arg Ser Lys Leu Pro Asp Ile Thr Ile Ser Asn Asp Leu Pro Leu His
 35 40 45
 gca tac tgc ttt gaa aac ctc tct gat ttc tca gat agg cca tgc ttg 192
 Ala Tyr Cys Phe Glu Asn Leu Ser Asp Phe Ser Asp Arg Pro Cys Leu
 50 55 60
 att tca ggt tcc acg gga aaa acc tat tct ttt gcc gaa act cac ctc 240
 Ile Ser Gly Ser Thr Gly Lys Thr Tyr Ser Phe Ala Glu Thr His Leu
 65 70 75 80
 ata tct cgg aag gtc gct gct ggg tta tcc aat ttg ggc atc aag aaa 288
 Ile Ser Arg Lys Val Ala Ala Gly Leu Ser Asn Leu Gly Ile Lys Lys
 85 90 95
 ggc gat gta atc atg acc ctg ctc caa aac tgc cca gaa ttc gtc ttc 336
 Gly Asp Val Ile Met Thr Leu Leu Gln Asn Cys Pro Glu Phe Val Phe
 100 105 110
 tcc ttc atc ggt gct tcc atg att ggt gca gtc atc acc act gcg aac 384
 Ser Phe Ile Gly Ala Ser Met Ile Gly Ala Val Ile Thr Thr Ala Asn
 115 120 125
 cct ttc tac act caa agt gaa ata ttc aag caa ttc tct gct tct cgt 432
 Pro Phe Tyr Thr Gln Ser Glu Ile Phe Lys Gln Phe Ser Ala Ser Arg
 130 135 140
 gcg aaa ctg att atc acc cag tct caa tat gtg aac aag cta gga gat 480
 Ala Lys Leu Ile Ile Thr Gln Ser Gln Tyr Val Asn Lys Leu Gly Asp
 145 150 155 160
 agt gat tgc cat gaa aac aac caa aaa ccg ggg gaa gat ttc ata gta 528
 Ser Asp Cys His Glu Asn Asn Gln Lys Pro Gly Glu Asp Phe Ile Val
 165 170 175
 atc acc att gat gac ccg cca gag aac tgt cta cat ttc aat gtg ctt 576
 Ile Thr Ile Asp Asp Pro Pro Glu Asn Cys Leu His Phe Asn Val Leu

180										185					190					
gtc	gag	gct	agc	gag	agt	gaa	atg	cca	aca	ggt	tca	atc	ctt	ccg	gat	624				
Val	Glu	Ala	Ser	Glu	Ser	Glu	Met	Pro	Thr	Val	Ser	Ile	Leu	Pro	Asp					
		195					200					205								
gat	cct	gtg	gca	tta	cca	ttc	tct	tca	ggg	aca	aca	ggg	ctc	cca	aaa	672				
Asp	Pro	Val	Ala	Leu	Pro	Phe	Ser	Ser	Gly	Thr	Thr	Gly	Leu	Pro	Lys					
	210					215					220									
gga	gtg	ata	ctg	acc	cac	aag	agc	ttg	ata	aca	agt	gtg	gct	caa	caa	720				
Gly	Val	Ile	Leu	Thr	His	Lys	Ser	Leu	Ile	Thr	Ser	Val	Ala	Gln	Gln					
225					230					235					240					
gtt	gat	gga	gag	atc	cca	aat	tta	tac	ttg	aaa	caa	gat	gac	gtt	gtt	768				
Val	Asp	Gly	Glu	Ile	Pro	Asn	Leu	Tyr	Leu	Lys	Gln	Asp	Asp	Val	Val					
				245					250					255						
tta	tgc	gtt	tta	cct	ttg	ttt	cac	atc	ttt	tca	ttg	aac	agc	gtg	ttg	816				
Leu	Cys	Val	Leu	Pro	Leu	Phe	His	Ile	Phe	Ser	Leu	Asn	Ser	Val	Leu					
			260					265					270							
tta	tgc	tcg	ttg	aga	gcc	ggg	tct	gct	gtt	ctt	tta	atg	caa	aag	ttt	864				
Leu	Cys	Ser	Leu	Arg	Ala	Gly	Ser	Ala	Val	Leu	Leu	Met	Gln	Lys	Phe					
		275					280					285								
gag	ata	gga	tca	ctg	cta	gag	ctc	att	cag	aaa	cac	aat	gtt	tcg	gtt	912				
Glu	Ile	Gly	Ser	Leu	Leu	Glu	Leu	Ile	Gln	Lys	His	Asn	Val	Ser	Val					
	290					295					300									
gcg	gct	gtg	gtg	cca	cca	ctg	gtg	ctg	gcg	ttg	gcc	aag	aac	cca	ttg	960				
Ala	Ala	Val	Val	Pro	Pro	Leu	Val	Leu	Ala	Leu	Ala	Lys	Asn	Pro	Leu					
305					310					315					320					
gag	gcg	aac	ttc	gac	ttg	agt	tcg	atc	agg	gta	gtc	ctg	tca	ggg	gct	1008				
Glu	Ala	Asn	Phe	Asp	Leu	Ser	Ser	Ile	Arg	Val	Val	Leu	Ser	Gly	Ala					
				325					330					335						
gcg	cca	ctg	ggg	aag	gag	ctc	gag	gac	gcc	ctc	agg	agc	agg	gtt	cct	1056				
Ala	Pro	Leu	Gly	Lys	Glu	Leu	Glu	Asp	Ala	Leu	Arg	Ser	Arg	Val	Pro					
			340					345					350							
cag	gcc	atc	ctg	gga	cag	ggt	tat	ggg	atg	aca	gag	gcc	ggg	cct	gtg	1104				
Gln	Ala	Ile	Leu	Gly	Gln	Gly	Tyr	Gly	Met	Thr	Glu	Ala	Gly	Pro	Val					
		355					360					365								
cta	tca	atg	tgc	tta	gcc	ttt	tca	aag	caa	cct	ttc	cca	acc	aag	tct	1152				
Leu	Ser	Met	Cys	Leu	Ala	Phe	Ser	Lys	Gln	Pro	Phe	Pro	Thr	Lys	Ser					
		370				375					380									
ggg	tcg	tgt	gga	acg	gtg	gtt	aga	aac	gca	gag	ctc	aag	gtc	att	gac	1200				
Gly	Ser	Cys	Gly	Thr	Val	Val	Arg	Asn	Ala	Glu	Leu	Lys	Val	Ile	Asp					
385																				

[illegible][illegible][illegible][illegible][illegible][illegible][illegible]

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)	(31)	(32)	(33)	(34)	(35)	(36)	(37)	(38)	(39)	(40)	(41)	(42)	(43)	(44)	(45)	(46)	(47)	(48)	(49)	(50)	(51)	(52)	(53)	(54)	(55)	(56)	(57)	(58)	(59)	(60)	(61)	(62)	(63)	(64)	(65)	(66)	(67)	(68)	(69)	(70)	(71)	(72)	(73)	(74)	(75)	(76)	(77)	(78)	(79)	(80)	(81)	(82)	(83)	(84)	(85)	(86)	(87)	(88)	(89)	(90)	(91)	(92)	(93)	(94)	(95)	(96)	(97)	(98)	(99)	(100)
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	
101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)	(31)	(32)	(33)	(34)	(35)	(36)	(37)	(38)	(39)	(40)	(41)	(42)	(43)	(44)	(45)	(46)	(47)	(48)	(49)	(50)	(51)	(52)	(53)	(54)	(55)	(56)	(57)	(58)	(59)	(60)	(61)	(62)	(63)	(64)	(65)	(66)	(67)	(68)	(69)	(70)	(71)	(72)	(73)	(74)	(75)	(76)	(77)	(78)	(79)	(80)	(81)	(82)	(83)	(84)	(85)	(86)	(87)	(88)	(89)	(90)	(91)	(92)	(93)	(94)	(95)	(96)	(97)	(98)	(99)	(100)
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	
101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	
201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	
301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	
401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433																																																																				

[illegible][illegible][illegible]

```

      130              135              140
Ala Lys Leu Ile Ile Thr Gln Ser Gln Tyr Val Asn Lys Leu Gly Asp
145              150              155              160
Ser Asp Cys His Glu Asn Asn Gln Lys Pro Gly Glu Asp Phe Ile Val
      165              170              175
Ile Thr Ile Asp Asp Pro Pro Glu Asn Cys Leu His Phe Asn Val Leu
      180              185              190
Val Glu Ala Ser Glu Ser Glu Met Pro Thr Val Ser Ile Leu Pro Asp
      195              200              205
Asp Pro Val Ala Leu Pro Phe Ser Ser Gly Thr Thr Gly Leu Pro Lys
      210              215              220
Gly Val Ile Leu Thr His Lys Ser Leu Ile Thr Ser Val Ala Gln Gln
225              230              235              240
Val Asp Gly Glu Ile Pro Asn Leu Tyr Leu Lys Gln Asp Asp Val Val
      245              250              255
Leu Cys Val Leu Pro Leu Phe His Ile Phe Ser Leu Asn Ser Val Leu
      260              265              270
Leu Cys Ser Leu Arg Ala Gly Ser Ala Val Leu Leu Met Gln Lys Phe
      275              280              285
Glu Ile Gly Ser Leu Leu Glu Leu Ile Gln Lys His Asn Val Ser Val
      290              295              300
Ala Ala Val Val Pro Pro Leu Val Leu Ala Leu Ala Lys Asn Pro Leu
305              310              315              320
Glu Ala Asn Phe Asp Leu Ser Ser Ile Arg Val Val Leu Ser Gly Ala
      325              330              335
Ala Pro Leu Gly Lys Glu Leu Glu Asp Ala Leu Arg Ser Arg Val Pro
      340              345              350
Gln Ala Ile Leu Gly Gln Gly Tyr Gly Met Thr Glu Ala Gly Pro Val
      355              360              365
Leu Ser Met Cys Leu Ala Phe Ser Lys Gln Pro Phe Pro Thr Lys Ser
      370              375              380
Gly Ser Cys Gly Thr Val Val Arg Asn Ala Glu Leu Lys Val Ile Asp
385              390              395              400
Pro Glu Thr Gly Arg Ser Leu Gly Tyr Asn Gln Pro Gly Glu Ile Cys
      405              410              415
Ile Arg Gly Ser Gln Ile Met Lys Gly Tyr Leu Asn Asp Ala Glu Ala
      420              425              430
Thr Ala Asn Thr Ile Asp Val Glu Gly Trp Leu His Thr Gly Asp Ile
      435              440              445
Gly Tyr Val Asp Asp Asp Asp Glu Ile Phe Ile Val Asp Arg Val Lys
      450              455              460
Glu Ile Ile Lys Phe Lys Gly Phe Gln Val Pro Pro Ala Glu Leu Glu
465              470              475              480
Ala Leu Leu Val Asn His Pro Ser Ile Ala Asp Ala Ala Val Val Pro
      485              490              495
Gln Lys Asp Glu Val Ala Gly Glu Val Pro Val Ala Phe Val Val Arg
      500              505              510
Ser Asp Asp Leu Asp Leu Ser Glu Glu Ala Val Lys Glu Tyr Ile Ala
      515              520              525
Lys Gln Val Val Phe Tyr Lys Lys Leu His Lys Val Phe Phe Val His
      530              535              540
Ser Ile Pro Lys Ser Ala Ser Gly Lys Ile Leu Arg Lys Asp Leu Arg
545              550              555              560
Ala Lys Leu Ala Thr Ala Thr Thr Met Ser
      565              570

```

<210> 5

<211> 1172

<212> DNA

<213> Populus tremuloides Michx. (aspen)

<400> 5

tgtaggattg	gtggaatggg	atcattccta	atcccttaat	gacgggtggca	tgaacacaaa	60
gcaaagagaa	gttaggtcac	tcctccttta	tatatatata	tatatgcatg	catgaggacc	120
atggctatga	tgaaggttaa	tagaggtagt	tgtgattgag	atatgtccag	cactagtttt	180
ttgttggtgt	gatttctcat	gatgacgcga	aaattttata	tatatatata	atgaataata	240
tgattgatta	ttctctgtaa	ttttgtgaaa	tagattaaaa	cagctcaatg	tgaggtgacc	300
agttgtcaaa	tgaccactcg	acttggggca	tgggtgatttt	tcaaatacaca	actcaatttg	360
aaaactaaaa	ttaaaaaaga	tttagattat	taaattatta	ggttaattca	cgggttggct	420
aatcaattat	tattaattaa	aacgatagta	tttttgataa	tttaattaaa	attttattgg	480
atttgaatga	actcaattac	atcacaaaaa	acctaataca	attaatatct	tatgtgatat	540
aatttagaaa	tataaatgat	taacctttaa	atctcgagtt	tctcttataa	aaaacacgta	600
taattgggct	agatttaaca	gctattattc	aaactggcca	ggacaattat	taaaattaat	660
aattattatt	ttttctaata	aagcacttcc	taattgttaa	aatatatgtc	taaacactaa	720
taataaaaatt	tatttgtgta	tctttggcag	taggtgagag	gtgctgacaa	ataaattagt	780
gcataaaaata	taatggattg	gtggtctgtg	aaaagacagg	tggaggacaa	gccacctctc	840
tcaagtcaaa	aggccatttc	acaaccaacc	caaattggga	cccaccaccg	ttccccgcc	900
ttaaaatccc	taatctcacc	aacccaactc	cacagattct	tcaccaaacg	caactgattt	960
ttcaatcaat	gttttcccta	tactaccccc	ccaacaactc	cataataccc	aatttgcctt	1020
ttcaccaaac	ccgtcctcc	gtgccagcca	attctatata	agcaggaatg	ctctgcactc	1080
tgctttctca	ggctccttac	cataagaaaa	cagagagcac	ctaaaactcg	ccatctctcc	1140
ctctgcatct	ttagcccgca	atggacgcga	ca			1172

<210> 6

<211> 1180

<212> DNA

<213> Populus tremuloides Michx. (aspen)

<400> 6

aagctttgag	tattcatatg	ggtattcatc	cgaccattat	ttttcaattt	gtgttgtggt	60
gatccaattt	tcaacttatt	tttttttcac	ttatttttta	ttagttattt	ttatttttat	120
tattttttta	aaaattttaa	aattaaatta	taacattttt	attttatccc	tcattaacta	180
aaatagggat	ggtaatatag	attcatgaag	ggagttatat	atcaaataat	attagtttaag	240
ctattttgat	atttataccc	tactcattac	ttatggaata	aaaaatttag	atattttata	300
aatattttatc	ggatttcagg	tattcatatg	aatattttat	tgattattat	ttattcaaca	360
aaaaataaaa	caattaatat	gcatgtttga	agttttatata	tatattaagt	taggttttaga	420
tagatttttg	gtggggttaa	ttaatattca	taccctatct	actatctatc	aaataatcca	480
aataaaattca	cctaaattag	gttgggtttg	tattcatcaa	gttaacatta	aattgtaatt	540
ccgtaaagtaa	ctaaacaagt	acaaagactt	ctattttatc	ttatatatta	ccataaaagcc	600
aactatattt	cctattcttt	ttcatccctt	ctatcgtaat	tttctgtgac	ttttttattt	660
atatattaac	ggtaacgaaa	cacagcaata	aaagttattg	tgaaagatat	ggataattat	720
tatggtgact	atgaaagagt	aaatttgcca	tgcactaagt	tcctagtgtc	atctcataaa	780
agacttgctc	gccacgtaag	ctggttgtag	tgtcgtttat	ttacgcgtgt	caaccaatcg	840
ctgccaatg	actcttgagg	gtaggtgaga	gcttcggcct	tgatgggaac	tgcatgaggc	900
atagggtttg	gtttcttgaa	tgtgagatgg	gcatgctttg	gctcccttgc	tactcacctc	960
atcttcaatt	tgccagctca	gctaccagtc	tctcaccact	agtttcacca	aactttctct	1020
gctcctgtat	ttattacacc	ttgctcgatt	ggctccgtcc	tcgtacacgc	atccacaccg	1080
atcgatcgat	tagaaccata	cagaattggg	attggttggg	tttaccattct	gcgttagata	1140
catctatcac	agaaagaaac	tcccttccat	ctcaggaaac			1180

<210> 7

<211> 12

<212> PRT

<213> Populus tremuloides Michx. (aspen)

<400> 7

Leu	Pro	Tyr	Ser	Ser	Gly	Thr	Thr	Gly	Leu	Pro	Lys
1					5						10

<210> 8

<211> 7

<400> 15
cctttcacca accccc 16

<210> 16
<211> 6
<212> DNA
<213> Populus tremuloides Michx. (aspen)

<400> 16
ccgttc 6

<210> 17
<211> 11
<212> DNA
<213> Populus tremuloides Michx. (aspen)

<400> 17
tctcaccaac c 11

Populus tremuloides Michx. (aspen)